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(54) Title: IDENTIFICATION OF FACTORS WHICH MEDIATE THE INTERACTION OF HETEROTRIMERIC G PROTEINS AND MONOMERIC G PROTEINS

(57) Abstract

Monomeric GTPase guanine nucleotide exchange factor (GEF) have been identified which also contain an RGS region analogous to those of GTPase activating proteins (GAP). One of these GEF proteins, a Rho GEF has been demonstrated to contain an RGS sequence that has GAP activity toward a α subunit of a heterotrimeric G prote in.

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IDENTIFICATION OF FACTORS WHICH MEDIATE THE INTERACTION OF HETEROTRIMERIC G PROTEINS AND MONOMERIC G PROTEINS

BACKGROUND OF THE INVENTION

Signal transduction pathways linking extracellular factors to the activation of the Rho GTPase have been implicated in cell growth control and cytoskeletal rearrangements. Specifically, heterotrimeric G proteins have been shoαwn to mediate these pathways, although the mechanism of mediation has been unclear. The identification of factors which interact with both heterotrimeric G proteins and Rho GTPase would provide an important tool for investigating and controlling various cell processes, including cell proliferative diseases.

SUMMARY OF THE INVENTION

The invention relates to a polypeptide, and corresponding nucleic acid. comprising an amino acid sequence of a novel RGS domain, obtainable, e.g., from a guanine nucleotide exchange factor (GEF) protein, where the polypeptide preferably does not include a dbl homology (DH) domain or a pleckstrin homology (PH) domain. In a preferred embodiment, the polypeptide has GTPase activating activity and binding affinity for an a G protein subunit such as Gα.

The polypeptides and nucleic acids can be used as tools for research, therapeutics, and diagnostics as discussed below.

The invention also relates to a method of identifying or assaying for a molecule, or mixture of molecules, that regulate the binding of an RGS domain of a GEF protein to a substrate, e.g., a G protein subunit such as Gα . In one embodiment, the method involves incubating, under effective conditions, a polypeptide having an RGS domain of a GEF polypeptide, and optionally having GEF activity, with a Gα subunit, or a fragment thereof, in the presence and/or absence of a test molecule; and determining whether the presence of the test molecule regulates the binding between the polypeptide and the subunit, or fragment thereof. As discussed later, various RGS-GEF polypeptides binding substrates can be utilized.

In addition, the invention relates to a method of identifying or assaying for a molecule, or mixture of molecules, that regulates a stimulatory effect of a polypeptide

comprising an RGS domain of a GEF protein on a polypeptide having a GTPase activity. In a preferred embodiment, the method comprises incubating a Gα subunit and a GEF protein, under effective conditions, in the presence and absence of a test molecule and determining whether the presence of the test molecule regulates the stimulatory effect of the GEF protein on Gα subunit GTPase activity.

The invention also relates to a method of identifying or assaying for a molecule that specifically regulates a stimulatory effect of a first polypeptide, such as an activated Gα subunit, or polypeptide having GTPase activity, on a nucleotide exchange factor activity of a second polypeptide. The second polypeptide preferably comprises a RGS-GEF domain obtainable from a GEF, and more preferably is a guanine nucleotide exchange factor (GEF) for a monomeric G protein. In one embodiment of the method, a first assay is conducted by incubating an activated Gα subunit with a GEF protein and a monomeric G protein in the presence and absence of a test molecule; a second assay is conducted: by incubating a GEF protein and a monomeric G protein in the presence and absence of the test molecule, and a determination is made as to whether the molecule has a different effect when the first assay is compared to the second assay.

The invention further relates to a method of identifying or assaying for a molecule, or mixture of molecules, that mimics the stimulatory effect of an activated Gα subunit on GEF mediated nucleotide exchange of a monomeric G protein. In one example, such a method comprises identifying a test compound that exhibits a binding affinity for an RGS domain of GEF proteins, incubating a GEF protein and monomeric G protein in the presence or absence of the test compound, determining whether the test compound exhibits a stimulatory effect on GEF mediated nucleotide exchange of a monomeric G protein.

The invention further relates to a method of identifying or assaying for a molecule, or mixture of molecules, that mimics the stimulatory effect of an RGS domain of GEF polypeptide on Gα subunit GTPase activity. In one example, such a method comprises identifying a test compound that exhibits a binding affinity for a Gα subunit and incubating a GTP loaded Gα subunit in the presence or absence of the test compound to determine whether the test compound exhibits a stimulatory effect on GEF mediated nucleotide exchange of a monomeric G protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1, Panel A depicts the alignment of the sequences from RGS proteins and the N-terminal region of p115 Rho GEF as performed by Clustal W with a secondary structure mask of RGS4 to assign penalties for gaps. The RGS homologous sequences of Lsc, KIAA380, and DrhoGEF2 were further added to this alignment by Clustal W and manual adjustments. The (a) symbols above RGS4 indicate the α helices of the RGS domain of RGS4. Dark shaded boxes indicate conserved residues of the hydrophobic core of the RGS structure. Lightly shaded boxes show other conserved residues. Asterisks mark the residues of RGS4 which contact $G\alpha_{i1}$. Primary sequences used in the alignment are the following: rat RGS4 (SwissProt accession number P49799), mouse RGS2 (O08849), human GAIP (P49795), rat RGS12 (O08774), rat RGS14 (O08773), human p115 (1654344), mouse Lsc (1389756), human KIAA380 (2224701) and Drosophila DrhoGEF2 (2760368).

Figure 1, Panel B depicts constructs of p115 Rho GEF that were employed in the studies described herein. Numbers indicate the residues of p115 in each construct. The RGS, dbl(DH), and pleckstrin (PH) homology regions are indicated. GST=glutathione-S-transferase.

Figure 2, Panel A is a graph showing the hydrolysis of GTP bound to $G\alpha_{13}$ and $G\alpha_{12}$ at 15°C either with (●○) or without (■□) 10 nM p115 Rho GEF.

Figure 2, Panel B is a graph showing the hydrolysis at 4°C of GTP bound to $G\alpha_{13}$ (●) and $G\alpha_{12}$ (○) and in the presence of various concentrations of p115 Rho GEF. The initial rates of reaction were plotted as a function of the concentration of p115 Rho GEF.

Figure 3 is a graph showing the hydrolysis at 15°C of GTP bound to $G\alpha_{13}$ and $G\alpha_{12}$ with either full-length p115 Rho GEF (●), ΔNp115 (■), or RGS-p115 (▲), or without any p115 construct (▼).

Figure 4 is a graph showing the hydrolysis of GTP bound to $G\alpha_{i1}$, $G\alpha_z$, $G\alpha_q$, and $G\alpha_s$ with 100 nM p115 Rho GEF (Δ), 100 nM RGS4 (□), or buffer control (○). Assays were performed at 4°C for $G\alpha_{i1}$ and $G\alpha_s$, at 15°C for $G\alpha_z$, and at 20°C for $G\alpha_q$.

Figure 5 is a graph showing the selective inhibition of p115 GAP activity by the AlF_4- activated forms of Gα subunits. Panel A: P115 (400 nM) was incubated on ice for 15 minutes with various Gα subunits (400 nM) in the presence of 30 μM $AlCl_3$, 10 mM NaF,

and 10 mM $MgSO_4$. The mixture was diluted 20-fold, mixed with 0.3 nM $G\alpha_{12}$(GTP) and the hydrolysis of bound GTP was measured after incubation at 15°C for 2 minutes. Panel B: P115 (400 nM) was incubated with various concentrations of $G\alpha_{12}$(GDP-AlF_4^-) (●) or $G\alpha_{13}$(GDP-AlF_4^-) (■) as described for Panel A. The mixture was diluted 20-fold, mixed with 1nM $G\alpha_{13}$(GTP) at 4°C and the hydrolysis of bound GTP was assessed over time. The initial rate of GTPase of $G\alpha_{13}$ was plotted against the final concentration of α subunit GDP-AlF_4^-. The filled triangle indicates the rate of GTPase of $G\alpha_{13}$ without p115.

Figure 6, Panel A is an image of an immunoblot showing the detection of myc-tagged p115 Rho GEF expression in COS cells using an anti-myc antibody.

Figure 6, Panel B is an image of an immunoblot showing the detection of a coimmunoprecipitate of p115 Rho GEF and $G\alpha_{13}$ using an anti-myc antibody.

Figure 6, Panel C is an image of an immunoblot showing the detection of the coimmunoprecipitate of p115 Rho GEF and $G\alpha_{13}$ using an anti-$G\alpha_{13}$ antibody.

Figure 6, Panel D is an image of an immunoblot showing the detection of p115 Rho GEF and $G\alpha_{13}$ binding when purified $G\alpha_{13}$ is added to immunoprecipitated p115 Rho GEF when using an anti $G\alpha_{13}$ antibody.

Figure 7, Panel A is a graph showing the dissociation of bound GDP from 100 nM RhoA after 10 minutes in the presence or absence of 100 nm $G\alpha_{13}$ or $G\alpha_{12}$ and in the presence of various concentrations of p115 Rho GEF as indicated.

Figure 7, Panel B is a graph showing the dissociation of GDP from 100 nM RhoA after 10 minutes in the presence of 25 nm p115 Rho GEF and the indicated concentrations of $G\alpha_{13}$ or $G\alpha_{12}$. Unstimulated dissociation of GDP from RhoA is indicated by the lower dashed line.

Figure 7, Panel C is a graph showing the dissociation of GDP from 100 nM RhoA after 10 minutes of incubation with p115 Rho GEF and $G\alpha_{13}$ that had been treated with AMF, GTPγS or GDPβS as indicated.

Figure 7, Panel D is a graph showing the dissociation of of GDP from 100 nM RhoA after 10 minutes of incubation with p115 Rho GEF (25 nM) and various Gα subunits (100 nM) as indicated.

Figure 8, Panel A is a graph showing the association of 1 nM [^{32}P]GTP to 100 nM RhoA in the presence of the indicated concentrations of truncated for full-length p115 Rho GEF as measured by filtration after 30 minutes at 30°C.

Figure 8, Panel B is a graph showing the dissociation of [^{3}H]-GDP from 100 nM RhoA after incubation for 10 minutes in the presence or absence of 25 nM p115 Rho GEF, 20 nM $G\alpha_{13}$, and 300 nM GST-RGSp115 as indicated.

Figure 8, Panel C is a graph showing the dissociation of [^{3}H]-GDP from 100 nM RhoA after incubation for 10 minutes in the presence 25 nM p115 Rho GEF and in the presence or absence of 25 nM $G\alpha_{13}$ and the indicated concentrations of $G\alpha_{12}$.

Figure 9, Panel A is an image of an immunoblot showing the detection of myc-tagged KIAA380 (designated FL147) expression in COS cells using an anti-myc antibody.

Figure 9, Panel B is an image of an immunoblot showing the detection of a coimmunoprecipitate of KIAA380 (designated FL147) and $G\alpha_{12}$ using an anti-$G\alpha_{12}$ antibody.

Figure 10 is the a listing of the amino acid sequence for p115 Rho GEF.. The RGS domain is shown by amino acids 45-170.

Figure 11 is a listing of the nucleic acid sequence for p115 Rho GEF. The RGS domain is encoded by nucleotides 187-564.

Figure 12 is a listing of the amino acid sequence for KIAA380. The RGS domain is shown by amino acids 310-432.

Figure 13 is a listing of the nucleic acid sequence for KIAA380. The RGS domain is encoded by nucleotides 1673-2041.

Figure 14 is a listing of the amino acid sequence for Lsc. The RGS domain is shown by amino acids 43-168.

Figure 15 is a listing of the nucleic acid sequence for Lsc. The RGS domain is encoded by nucleotides 218-595.

Figure 16 is a listing of the amino acid sequence for DRhoGEF2. The RGS domain is shown by amino acids 924-1053

Figure 17 is a listing of the nucleic acid sequence for DRhoGEF2. The RGS domain is encoded by nucleotides 3185-3574.

Figure 18 is a homology alignment of the RGS region of several proteins, including GEF proteins with RGS domains (e.g. p115 Rho GEF, Lsc, KIAA380, DrhoGEF). The alignment was performed using the Clustal method with a PAM250 residue weight table.

DETAILED DESCRIPTION OF THE INVENTION

G proteins transduce signals from a large number of cell surface heptahelical receptors to various intracellular effectors. Each heterotrimeric G protein is composed of a guanine nucleotide-binding α subunit and a high-affinity dimer of β and γ subunits. Gα subunits are commonly classified into four subfamilies (G_s, G_i, G_q, and G_{12}) based on their amino acid sequence homology and function (A.G. Gilman, *Annu. Rev. Biochem*, **56**, 615 (1987); Y. Kaziro et al., *Annu. Rev. Biochem.*, **60**, 349 (1991); Hepler and Gilman, *Trends Biochem. Sci.*, **17**, 383, (1992)). The G_{12} subfamily, consists of two identified members to date, G_{12} and G_{13}.

In accordance with the present invention, the identification of proteins having activity as both a GTPase activating protein (GAP) for the α subunit of a heterotrimeric G protein and activity as a guanine nucleotide exchange factor (GEF) activity for monomeric G proteins have been described. Also in accordance with the invention, the first identification of a protein having GAP activity for the G_{12} subfamily of G proteins has been described. Also in accordance with the invention, the ability of an α subunit of a heterotrimeric G protein to stimulate GEF mediated guanine nucleotide exchange activity of a monomeric G protein has been described. GAP and GEF activity, and methods of screening thereof, are described in Berman et al., 1996, *Cell* **86**:445 and Hart et al., 1996, *J. Biol. Chem.*, **271**:25452.

According to the present invention, the GAP activity of GEF proteins has been correlated with a novel RGS domain obtainable from a GEF protein. The present invention relates to all aspects of such an RGS domain, including all aspects of a Rho GEF such as p115 Rho-GEF. (U.S. Patent Application No. 08/943,768, herein incorporated by reference).

A GEF protein modulates cell signaling pathways, both in *in vitro* and *in vivo*, by modulating the guanine nucleotide exchange activity of a GTPase. According to the present invention, a GEF protein which also modulates the GTPase activity of a heterotrimeric Gα subunit is described. By way of illustration, p115 Rho-GEF, which modulates the guanine

nucleotide exchange activity of a Rho GTPase, as well as the GTPase activity of the $G\alpha_{12}$ family of heterotrimeric G protein subunits is described.

The present invention particularly relates to polypeptides comprising a RGS domain of a GEF polypeptide, or fragments thereof, and corresponding nucleic acids.

The invention also relates to methods of using such polypeptides, nucleic acids, or derivatives thereof, e.g., in therapeutics, diagnostics, and as research tools. Other aspects of the present invention relate to antibodies and other ligands which recognize the RGS domain of GEF polypeptides or nucleic acids, methods for identifying or assaying modulators of the GEF activities and/or the GAP activities of a protein containing a RGS domain, and methods of treating pathological conditions associated with or related to the RGS domain, e.g., a GEF mediated interaction of a Gα subunit and a Rho GTPase.

As used herein, an "RGS-GEF polypeptide" means, e.g., a polypeptide containing an RGS domain derived from a GEF protein, such as p115 Rho-GEF, Lsc, KIAA0380, or DRhoGEF2, and, which has one or more of the following activities: a specific binding affinity for a polypeptide substrate, e.g., a G protein subunit, preferably an α subunit, such as G_{12} or G_{13}; a GTPase activating activity (GAP), such as a GAP activity for a G protein α subunit; or, an immunogenic activity. An RGS-GEF polypeptide preferably does not contain a (dbl homology) DH or a (pleckstrin homology) PH domain. DH and PH domains are disclosed in Cerione and Zheng, 1996, *Curr. Opin. In Cell Biol.*, **8**:216. For example, the amino acid sequence of p115 Rho GEF (Fig.10) contains a novel RGS domain at amino acids 45-170, the DH domain at amino acids 420-637, and the PH domain at amino acids 646-672. By "derived," it is meant that the amino acid sequence is obtainable from a naturally-occurring GEF (such as p115, Lsc, KIAA380, and DrrhoGEF2) or a non-naturally-occurring "mutated" sequence which is based upon a naturally-occurring GEF sequence (i.e., different amino acid residues have been substituted for the amino acid residues which occur in the naturally-occurring sequence at a particular position). The polypeptide can be "isolated," i.e., the material is in a form in which it is not found in its original environment, e.g., more concentrated, more purified, or separated from other components,etc. A preferred RGS polypeptide possesses both a GAP and GEF activity, e.g., a mutated p115 Rho-GEF. See below.

An RGS-GEF nucleic acid codes for an RGS-GEF polypeptide. The nucleic acid refers to both sense and anti-sense nucleic acids.

By the term "specific binding affinity," it is meant, e.g., that the RGS-GEF polypeptide has a binding preference for the activated state or transition state of a G protein subunit as compared to a GDP-bound state or the nucleotide depleted state. By "GEF activity," it is meant, e.g., that the polypeptide stimulates or catalyzes the dissociation of GDP from a monomeric G-protein, such as Rho, and subsequent binding of GTP. Monomeric G-proteins include but are not limited to G-proteins in the Ras, Rho/Rac, Sar, Rab, Arf, and Ran families. Of particular interest are the RGS domains of the following GEF proteins: human p115 (1654344) (Fig. 10, RGS domain at amino acids 45-170), mouse Lsc (1389756) (Fig. 14, RGS domain at amino acids 43-168), KIAA380 (2224701) (Fig. 12, RGS domain at amino acids 310-432) and Drosophila DrhoGEF2 (2760368) (Fig. 16, RGS domain at amino acids 924-1053).

Another aspect of the invention relates to novel consensus sequences for RGS domain(s) of a GEF protein, herein referred to as a "sub-RGS consensus sequence." An "RGS domain," as used herein, refers to the amino acid sequence of protein which is able to bind to or physically interact with a G protein and, optionally, stimulates GTPase activity of that protein. A "sub-RGS consensus sequence," as used herein, refers to a consensus sequence which can be used to identify a specific subset of proteins which contain an RGS domain. For example, a homology alignment of the RGS domain from several proteins as shown and described in Fig. 18 and the corresponding legend, shows that several sub-RGS consensus sequences may be defined by the gap of 13 to 14 amino acids that is apparent in the RGS domains of GEF proteins. One of these consensus sequences, herein designated as "RGS-GEF consensus 1," is herein defined to be a consensus sequence of AA_1-AA_2-AA_3-AA_4-AA_5-AA_6-AA_7-AA_8-(gap of 13 amino acids)-AA_{22} -AA_{23}-AA_{24}-AA_{25}-AA_{26}, wherein:

AA_1 is L;

AA_2 is E or V;

AA_3 is K or P;

AA_4 is T, N, or R;

AA_5 is A;

AA_6 is V or P

AA_7 is L;

AA_8 is either S or a gap of one amino acid, contiguous with the gap of 13 amino acids;

AA_{22} is either R or W;

AA_{23} is either V or Y;

AA_{24} is either P,K, or R

AA_{25} is either V, I, or Q;

AA_{26} is either P or D.

A second consensus sequence, herein designated as "RGS-GEF consensus 2," is herein defined to be a consensus sequence of AA_1-AA_2-AA_3-AA_4-(gap of 13 amino acids)-AA_{18} -AA_{19}, wherein:

AA_1 is A;

AA_2 is V or P;

AA_3 is L;

AA_4 is either S or a gap of one amino acid, contiguous with the gap of 13 amino acids;

AA_{18} is either R or W;

AA_{19} is either V or Y.

Other proteins, including other GEF proteins can be aligned with the RGS domain of RGS proteins as shown in Figure 18, and using methods described herein, to determine if they contain a sub-RGS consensus sequence, such as RGS-GEF consensus 1 or RGS-GEF consensus 2, as defined above.

In examining Figure 18 it is also apparent that a nucleotide sequence uniques to RGS proteins that are not GEF proteins is shown by the nucleotide sequences which encode the amino acids that correspond to the 13-14 amino acid gap in RGS-GEF proteins. These nucleotide sequences could be used as probes to identify particular types of RGS proteins.

RGS-GEF polypeptides are preferably biologically-active. By biologically-active, it is meant that a polypeptide fragment possesses an activity in a living system or with component(s) of a living system. Biological-activities include, but are not limited to a specific binding affinity for a G protein α subunit, as defined above, and GAP activity toward a G protein α subunit. As described in the examples, such polypeptides can be prepared routinely, e.g., by recombinant means or by proteolytic cleavage of isolated polypeptides, and then assayed for a desired activity.

A polypeptide of the present invention includes polypeptides which have less than 100% identity to the amino acid sequences of p115 Rho-GEF (Fig. 10), Lsc (Fig. 14), KIAA0380 (Fig. 12), or DRhoGEF2 (Fig. 16). For the purposes of the following discussion: Sequence identity means that the same nucleotide or amino acid which is found in the sequences set forth in Fig. 10-17 is found at the corresponding position of the compared sequence(s). A polypeptide having less than 100% sequence identity to the amino acid sequences set forth in Figures 10, 12, 14, and 16 can be substituted in various ways, e.g., by a conservative amino acid. The sum of the identical and conservatively substituted residues divided by the total number of residues in the sequence is equal to the percent sequence similarity. For purposes of calculating sequence identity and similarity, the compared sequences can be aligned and calculated according to any desired method, algorithm, computer program, etc., including, e.g., FASTA, BLASTA. A polypeptide having less than 100% amino acid sequence identity to the amino acid sequences of the GEF proteins shown in Figures 10, 12, 14, and 16 may comprise, for example, about 60, 65 percent sequence similarity and more preferably about 67, 70, 78, 80, 90, 92, 96, 99, etc. percent sequence amino acid sequence similarity.

In particular, the present invention relates to polypeptides, and corresponding nucleic acids, of p115, Lsc, KIAA380, and DrhoGEF2 which are mutated in the RGS domain of a GEF protein and which possess one or more of the RGS-GEF polypeptide activities mentioned above. By the term "mutated," it is meant herein that such sequences are not naturally-occurring. For example a mutated polypeptide as mentioned can have one or more naturally-occurring positions replaced by a conservative amino acid, e.g., (based on the size of the side chain and degree of polarization) small nonpolar: cysteine, proline, alanine, threonine; small polar: serine, glycine, aspartate, asparagine; large polar: glutamate, glutamine, lysine, arginine; intermediate polarity: tyrosine, histidine, tryptophan; large nonpolar: phenylalanine, methionine, leucine, isoleucine, valine. Such conservative substitutions also include those described by Dayhoff in the Atlas of Protein Sequence and Structure 5 (1978), and by Argos in EMBO J., 8, 779-785 (1989). A polypeptide having an amino acid sequence as set forth in Figures 10, 12, 14, and 16 can be substituted at 1, 5, 10, 15, or 20 positions by conservative amino acids. The mutations can be introduced into the conserved consensus region or the other residues of the RGS domain of a GEF protein.

A mutation to an RGS-GEF polypeptide can be selected to have one or more of the activities mentioned above, e.g., a specific binding affinity for a G protein α subunit, a GAP activity toward a G protein α subunit, etc. Assays for such activities can be conducted as described below or as disclosed in Cerione and Zheng, 1996, *Curr. Opin. In Cell Biol.*, **8**:216.

An RGS-GEF polypeptide can be modified by introducing amino acid substitutions into the hydrophobic core of the RGS domain (See Fig. 1, Panel A). For example, a conservative amino acid substitution would not be expected to affect activity, whereas as non-conservative amino acid substitution, e.g., changing a hydrophobic residue to a hydrophilic residue, would be expected to reduce or eliminate its activity. Hydrophobic resiudes are nonpolar amino acids such phenylalanine, leucine, isoleucine, valine, alanine, methionine, tryptophan, and cysteine. Hydrophilic residues are polar amino acids such as lysine, arginine, histidine, glutamate, and aspartate.

Modifications to a RGS-GEF polypeptide of the present invention or corresponding nucleotide sequence, e.g., mutations, can also be prepared based on homology searching from gene data banks, e.g., Genbank, EMBL. Sequence homology searching can be accomplished using various methods, including algorithms described in the BLAST family of computer programs, the Smith-Waterman algorithm, etc. For example, conserved amino acids can be identified between various sequences containing an RGS domain of various GEF proteins. (See Fig. 18) A mutation(s) can then be introduced into such sequences by identifying and aligning amino acids conserved between the polypeptides and then modifying an amino acid in a conserved or non-conserved position. A mutated RGS-GEF sequence can comprise conserved or non-conserved amino acids, e.g., between corresponding regions of homologous nucleic acids. For example, a mutated sequence can comprise conserved or non-conserved residues from any number of homologous sequences as mentioned-above and/or determined from an appropriate searching algorithm.

Corresponding mutations can be made in specific regions of an RGS-GEF nucleic acid. For example, mutations may be made wherein amino acids that particpate in the GTPase catalytic function or mutations may be made in amino acids that function as contact points between the RGS-GEF sequence and the Gα subunit.

An RGS-GEF polypeptide or fragment thereof, or substituted RGS-GEF polypeptide or fragment thereof, may also comprise various modifications, wherein such modifications include glycosylation, covalent modifications (e.g., of an R-group of an amino acid), amino acid substitution, amino acid deletion, or amino acid addition. Modifications to the polypeptide can be accomplished according to various methods, including recombinant, synthetic, chemical, etc.

Polypeptides of the present invention (e.g., RGS-GEF polypeptides, and fragments and mutations thereof) may be used in various ways, e.g., as immunogens for antibodies as described below, as biologically-active agents (e.g., having one or more of the activities associated with an RGS-GEF polypeptide), as inhibitors of the activities of the corresponding full-length polypeptide. For example, upon binding of p115 Rho-GEF to the Gα subunit, a cascade of events is initiated in the cell, e.g., promoting cell proliferation and/or cytoskeletal rearrangements. The interaction between p115 Rho-GEF and the Gα subunit can be modulated by using a RGS-GEF polypeptide, or fragment thereof, to inhibit the interaction between p115 Rho-GEF and the Gα subunit. Such a fragment can be useful for modulating pathological conditions associated with the Rho signaling pathway. A useful fragment may be identified routinely by testing the ability of overlapping fragments of the entire length of the RGS domain of a GEF protein to inhibit the binding of p115 Rho-GEF with the Gα subunit or to inhibit the GAP activity of the p115 Rho-GEF toward the Gα subunit. The measurement of these activities is described below and in the examples. Peptides can be chemically-modified, etc.

A RGS-GEF polypeptide of the present invention can comprise one or more structural domains, functional domains, detectable domains, antigenic domains, and/or other polypeptides of interest, in an arrangement which does not occur in nature, i.e., not naturally-occurring. A polypeptide comprising such features is a chimeric or fusion polypeptide. Such a chimeric polypeptide can be prepared according to various methods, including, chemical, synthetic, quasi-synthetic, and/or recombinant methods. A chimeric nucleic acid coding for a chimeric polypeptide can contain the various domains or desired polypeptides in a continuous or interrupted open reading frame, e.g., containing introns, splice sites, enhancers, etc. The chimeric nucleic acid can be produced according to various methods. See, e.g., U.S. Pat. No. 5,439,819. A domain or desired polypeptide can possess

any desired property, including, a biological function such as catalytic, signaling, growth promoting, cellular targeting, etc., a structural function such as hydrophobic, hydrophilic, membrane-spanning, etc., receptor-ligand functions, and/or detectable functions, e.g., combined with enzyme, fluorescent polypeptide, green fluorescent protein GFP (Chalfie et al., 1994, *Science*, 263:802; Cheng et al., 1996, *Nature Biotechnology*, 14:606; Levy et al., 1996, *Nature Biotechnology*, 14:610, etc. In addition, a RGS-GEF nucleic acid, or a fragment thereof, may be used as selectable marker when introduced into a host cell. For example, a nucleic acid coding for an amino acid sequence according to the present invention can be fused in frame to a desired coding sequence and act as a tag for purification, selection, or marking purposes. The region of fusion encodes a cleavage site.

A polypeptide according to the present invention can be produced in an expression system, e.g., in vivo, in vitro, cell-free, recombinant, cell fusion, etc., according to the present invention. Modifications to the polypeptide imparted by such system include, glycosylation, amino acid substitution (e.g., by differing codon usage), polypeptide processing such as digestion, cleavage, endopeptidase or exopeptidase activity, attachment of chemical moieties, including lipids, phosphates, etc. For example, some cell lines can remove the terminal methionine from an expressed polypeptide.

A polypeptide according to the present invention can be recovered from natural sources, transformed host cells (culture medium or cells) according to the usual methods, including, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, hydroxyapatite chromatography and lectin chromatography. It may be useful to have low concentrations (approximately 0.1-5 mM) of calcium ion present during purification (Price, et al., *J. Biol. Chem.*, 244:917 (1969)). High performance liquid chromatography (HPLC) can be employed for final purification steps.

A RGS-GEF nucleic acid of the present invention can comprise the complete coding sequence for an RGS-GEF polypeptide, or fragments thereof. A nucleic acid according to the present invention may also comprise a nucleotide sequence which is 100% complementary, e.g., an anti-sense, to any RGS-GEF nucleotide sequence.

A nucleic acid according to the present invention can be obtained from a variety of different sources. It may be obtained from DNA or RNA, such as polyadenylated mRNA,

e.g., isolated from tissues, cells, or whole organism. The nucleic acid may be obtained directly from DNA or RNA, or from a cDNA library. The nucleic acid can be obtained from a cell at a particular stage of development, having a desired genotype, phenotype (e.g., an oncogenically transformed cell or a cancerous cell), etc. The nucleic acid may also be chemically synthesized.

A nucleic acid according to the present invention may include only coding sequence for an RGS-GEF polypeptide; coding sequence for an RGS-GEF polypeptide and additional functional coding sequences including, for example, leader sequences, secretory sequences, tag sequences (e.g. targeting tags, enzymatic tags, fluorescent tags etc.). A nucleic acid according to the present invention may also include coding sequence for an RGS-GEF polypeptide and non-coding sequences, e.g., untranslated sequences at either a 5' or 3' end, or dispersed in the coding sequence, e.g., introns.

A nucleic acid according to the present invention may also comprise an expression control sequence operably linked to a nucleic acid as described above. The phrase "expression control sequence" means a nucleic acid sequence which regulates expression of a polypeptide coded for by a nucleic acid to which it is operably linked. Expression can be regulated at the level of the mRNA or polypeptide. Thus, the expression control sequence includes mRNA-related elements and protein-related elements. Such elements include promoters, enhancers (viral or cellular), ribosome binding sequences, transcriptional terminators, etc. An expression control sequence is operably linked to a nucleotide coding sequence when the expression control sequence is positioned in such a manner to effect or achieve expression of the coding sequence. For example, when a promoter is operably linked 5' to a coding sequence, expression of the coding sequence is driven by the promoter. Expression control sequences can be heterologous or endogenous to the normal gene.

A nucleic acid in accordance with the present invention may be selected on the basis of nucleic acid hybridization. The ability of two single-stranded nucleic acid preparations to hybridize together is a measure of their nucleotide sequence complementarity, e.g., base-pairing between nucleotides, such as A-T, G-C, etc. The invention thus also relates to nucleic acids which hybridize to a nucleic acids comprising a nucleotide sequence as set forth in Figures 11, 13, 15, and 17. The present invention includes both strands of nucleic acid, e.g., a sense strand and an anti-sense strand.

According to the present invention, a nucleic acid or polypeptide can comprise one or more differences in the nucleotide or amino acid sequence set forth in Figures 10-17. Changes or modifications to the nucleotide and/or amino acid sequence can be accomplished by any method available, including directed or random mutagenesis.

A nucleic acid coding for an RGS-GEF polypeptide according to the invention may comprise nucleotides which occur in a naturally-occurring GEF gene e.g., naturally-occurring polymorphisms, normal or mutant alleles (nucleotide or amino acid), mutations which are discovered in a natural population of mammals, such as humans, monkeys, pigs, mice, rats, or rabbits. By the term naturally-occurring, it is meant that the nucleic acid is obtained from a natural source, e.g., animal tissues and cells, body fluids, tissue culture cells, forensic samples. Naturally-occurring mutations include deletions, substitutions, or additions of nucleotide sequence. These genes can be detected and isolated by nucleic acid hybridization according to methods well known to one skilled in the art. It is recognized that, by analogy to other oncogenes, naturally-occurring variants of GEF proteins will include variants with deletions, substitutions, and additions in the RGS domain of a GEF protein, which produce pathological conditions in the host cell and organism.

A nucleotide sequence coding for an RGS-GEF polypeptide of the invention may contain codons found in a naturally-occurring gene, transcript, or cDNA, for example, or it may contain degenerate codons coding for the same amino acid sequences.

In addition, a nucleic acid or polypeptide of the present invention may be obtained from any desired mammalian organism, but also non-mammalian organisms. Homologs from mammalian and non-mammalian organisms can be obtained according to various methods. For example, hybridization with an oligonucleotide (see below) selective for an RGS domain of a GEF, or a RGS-GEF, of the present invention can be employed to select such homologs, e.g., as described in Sambrook et al., *Molecular Cloning*, 1989, Chapter 11. Such homologs may have varying amounts of nucleotide and amino acid sequence identity and similarity to previously identified RGS domain or RGS-GEF nucleotide or polypeptide sequence. Non-mammalian organisms include, e.g., vertebrates, invertebrates, zebra fish, chicken, *Drosophila*, yeasts (such as *Saccharomyces cerevisiae*), *C. elegans*, roundworms, prokaryotes, plants, *Arabidopsis*, viruses, etc.

A nucleic acid according to the present invention may comprise, for example, DNA, RNA, synthetic nucleic acid, peptide nucleic acid, modified nucleotides, or mixtures thereof. A DNA can be double- or single-stranded. Nucleotides comprising a nucleic acid can be joined via various known linkages such as, for example, ester, sulfamate, sulfamide, phosphorothioate, phosphoramidate, methylphosphonate, carbamate, etc., depending on the desired purpose. Linkages may be modified for purposes such as, for example, resistance to nucleases such as RNase H and improved *in vivo* stability. See, e.g., U.S. Pat. Nos. 5,378,825.

Various modifications can be made to the nucleic acids, such as attaching detectable markers (avidin, biotin, radioactive elements), moieties which improve hybridization, detection, or stability. The nucleic acids can also be attached to solid supports, e.g., nitrocellulose, nylon, agarose, diazotized cellulose, latex solid microspheres, polyacrylamides, etc., according to a desired method. See, e.g., U.S. Pat. Nos. 5,470,967, 5,476,925, 5,478,893.

Another aspect of the present invention relates to oligonucleotides and nucleic acid probes. Such oligonucleotides or nucleic acid probes can be used, e.g., to detect, quantify, or isolate an RGS-GEF nucleic acid in a test sample. Detection can be desirable for a variety of different purposes, including research, diagnostic, and forensic. For diagnostic purposes, it may be desirable to identify the presence or quantity of a specific RGS-GEF nucleic acid sequence in a sample obtained from tissues, cells, body fluids, etc. In a preferred method, the present invention relates to a method of detecting a target RGS-GEF nucleic acid in a test sample comprising contacting the test sample with an oligonucleotide under conditions effective to achieve hybridization between the target and oligonucleotide; and detecting hybridization. An oligonucleotide in accordance with the invention can also be used in synthetic nucleic acid amplification such as PCR, e.g., Saiki et al., 1988, Science, 241:53; U.S. Pat. No. 4,683,20, or or differential display (See, e.g., Liang et al., *Nucl. Acid. Res.*, 21:3269-3275, 1993; USP 5,599,672; WO97/18454). Oligonucleotides can be identified routinely, e.g., to the DH, PH, and RGS-GEF domains to differentially display and/or amplify gene products containing such sequences.

Both sense and antisense nucleotide sequences are intended as part of the invention. A unique nucleic acid according to the present invention may be determined routinely. An

RGS-GEF nucleic acid may be used as a hybridization probe to identify the presence of RGS-GEF nucleotide sequence in a sample comprising a mixture of nucleic acids, e.g., on a Northern blot. Hybridization can be performed under stringent conditions to select nucleic acids having at least 95% identity (i.e., complementarity) to the probe, but less stringent conditions can also be used. A unique RGS-GEF nucleotide sequence can also be fused in-frame, at either its 5' or 3' end, to various nucleotide sequences, including, for example, coding sequences for enzymes or expression control sequences, etc.

Hybridization can be performed under different conditions, depending on the desired selectivity, e.g., as described in Sambrook et al., *Molecular Cloning*, 1989. For example, to specifically detect RGS-GEF sequences, an oligonucleotide can be hybridized to a target nucleic acid under conditions in which the oligonucleotide only hybridizes to the GEF sequence from which the RGS -GEF sequence was derived, e.g., where the oligonucleotide is 100% complementary to the target. Different conditions can be used if it is desired to select target nucleic acids which have less than 100% nucleotide complementarity, at least about, e.g., 99%, 97%, 95%, 90%, 70%, 67%. Since a mutation in GEF genes can cause diseases or pathological conditions, e.g., cancer, benign tumors, an oligonucleotide according to the present invention can be used diagnostically. For example, a patient having symptoms of a cancer or other condition associated with the Rho signaling pathway (see below) can be diagnosed with the disease by using an oligonucleotide according to the present invention, in polymerase chain reaction followed by DNA sequencing to identify whether the sequence is normal, in combination with other oncogene oligonucleotides, etc., e.g., p53, Rb, p21, Dbl, MTS1, Wt1, Bcl-1, Bcl-2, MDM2, etc.

Oligonucleotides according to the present invention can be of any desired size, preferably 14-16 oligonucleotides in length, or more. Such oligonucleotides can have non-naturally-occurring nucleotides, e.g., inosine. In accordance with the present invention, the oligonucleotide can comprise a kit, where the kit includes a desired buffer (e.g., phosphate, tris, etc.), detection compositions, etc. The oligonucleotide can be labeled or unlabeled, with radioactive or non-radioactive labels as known in the art.

Anti-sense nucleic acid can also be prepared from a nucleic acid according to the present, preferably an anti-sense RGS-GEF nucleotide sequence corresponding to an RGS-GEF nucleotide sequence of Figures 11, 13, 15, and 17. Anti-sense RGS-GEF nucleic acid

can be used in various ways, such as to regulate or modulate expression of GEF proteins containing RGS domains or to detect expression of RGS-GEF proteins, including by *in situ* hybridization. For the purposes of regulating or modulating expression, an anti-sense oligonucleotide may be operably linked to an expression control sequence.

The RGS-GEF nucleic acids according to the present invention can be labelled according to any desired method. The nucleic acid can be labeled using radioactive tracers such as ^{32}P, ^{35}S, ^{125}I, ^{3}H, or ^{14}C, to mention only the most commonly used tracers. The radioactive labeling can be carried out according to any method such as, for example, terminal labeling at the 3' or 5' end using a radiolabeled nucleotide, polynucleotide kinase (with or without dephosphorylation with a phosphatase) or a ligase (depending on the end to be labeled). A non-radioactive labeling can also be used, combining a nucleic acid of the present invention with residues having immunological properties (antigens, haptens), a specific affinity for certain reagents (ligands), properties enabling detectable enzyme reactions to be completed (enzymes or coenzymes, enzyme substrates, or other substances involved in an enzymatic reaction), or characteristic physical properties, such as fluorescence or the emission or absorption of light at a desired wavelength, etc.

An RGS-GEF nucleic acid according to the present invention, including oligo-nucleotides, anti-sense nucleic acid, etc., can be used to detect expression of RGS-GEF nucleic acids in whole organs, tissues, cells, etc., by various techniques, including Northern blot, PCR, *in situ* hybridization, etc. Such nucleic acids can be particularly useful to detect disturbed expression, e.g., cell-specific and/or subcellular alterations of RGS-GEF expression. The levels of RGS-GEF proteins can be determined alone or in combination with other genes products (oncogenes such as p53, Rb, Wt1, etc.), transcripts, etc.

A nucleic acid according to the present invention can be expressed in a variety of different systems, *in vitro* and *in vivo*, according to the desired purpose. For example, a nucleic acid can be inserted into an expression vector, introduced into a desired host, and cultured under conditions effective to achieve expression of a polypeptide coded for the nucleic acid. Effective conditions includes any culture conditions which are suitable for achieving production of the polypeptide by the host cell, including effective temperatures, pH, medias, additives to the media in which the host cell is cultured (e.g., additives which amplify or induce expression such as butyrate, or methotrexate if the coding nucleic acid is

adjacent to a dhfr gene), cyclohexamide, cell densities, culture dishes, etc. A nucleic acid can be introduced into the cell by any effective method including, e.g., calcium phosphate precipitation, electroporation, injection, DEAE-Dextran mediated transfection, fusion with liposomes, and viral transfection. A cell into which a nucleic acid of the present invention has been introduced is a transformed host cell. The nucleic acid can be extrachromosomal or integrated into a chromosome(s) of the host cell. It can be stable or transient. An expression vector is selected for its compatibility with the host cell. Host cells include, mammalian cells, e.g., COS-7, CHO, HeLa, LTK, NIH 3T3, yeast, insect cells, such as Sf9 (*S. frugipeda*) and *Drosophila*, bacteria, such as *E. coli*, *Streptococcus sp.*, *Bacillus sp.*, yeast, fungal cells, plants, embryonic stem cells (e.g., mammalian, such as mouse or human), cancer or tumor cells Sf9 expression can be accomplished in analogy to Graziani et al., *Oncogene*, 7:229-235, 1992. Expression control sequences are similarly selected for host compatibility and a desired purpose, e.g., high copy number, high amounts, induction, amplification, controlled expression. Other sequences which can be employed include enhancers such as from SV40, CMV, inducible promoters, cell-type specific elements, or sequences which allow selective or specific cell expression.

A labelled polypeptide can be used, e.g., in binding assays, such as to identify substances that bind or attach to p115 Rho-GEF, to track the movement of p115 Rho-GEF in a cell, in an *in vitro*, *in vivo*, or *in situ* system, etc.

A nucleic acid or polypeptide of the present invention can also be substantially purified. By substantially purified, it is meant that nucleic acid or polypeptide is separated and is essentially free from other nucleic acids or polypeptides, i.e., the nucleic acid or polypeptide is the primary and active constituent.

Another aspect of the present invention relates to the regulation of biological pathways in which a RGS-GEF polypeptide is involved, particularly pathological conditions, e.g., cell proliferation (e.g., cancer), growth control, morphogenesis, stress fiber formation, and integrin-mediated interactions, such as embryonic development, tumor cell growth and metastasis, programmed cell death, hemostasis, leucocyte homing and activation, bone resorption, clot retraction, and the response of cells to mechanical stress. See, e.g., Clark and Brugge, Science, 268:233- 239, 1995; Bussey, Science, 272:225-226, 1996. Thus, the invention relates to all aspects of a method of modulating an activity of a RGS-GEF

polypeptide comprising, administering an effective amount of an RGS-GEF polypeptide or a biologically-active fragment thereof, an effective amount of a compound which modulates the activity of a RGS-GEF polypeptide, or an effective amount of a nucleic acid which codes for a RGS-GEF polypeptide or a biologically-active fragment thereof. The activity of the RGS-GEF which is modulated may include binding to a Gα subunit or GAP activity toward a Gα subunit. The activity can be modulated by increasing, reducing, antagonizing, or promoting expression or activity of the RGS-GEF.

The present invention also relates to antibodies which specifically recognize a RGS-GEF polypeptide. Antibodies, e.g., polyclonal, monoclonal, recombinant, chimeric, can be prepared according to any desired method. For example, for the production of monoclonal antibodies, an RGS-GEF polypeptide according to Figures 10, 12,14, or 16 can be administered to mice, goats, or rabbit subcutaneously and/or intraperitoneally, with or without adjuvant, in an amount effective to elicit an immune response. The antibodies can also be single chain or FAb. The antibodies can be IgG, subtypes, IgG2a, IgG1, etc.

An antibody specific for RGS-GEF means that the antibody recognizes a defined sequence of amino acids within or including the amino acid sequence of the RGS domain of a GEF polypeptide. Thus, a specific antibody will bind with higher affinity to an amino acid sequence, i.e., an epitope, found in the RGS domain of a GEF polypeptdie than to a different epitope(s), e.g., as detected and/or measured by an immunoblot assay. Thus, an antibody which is specific for an epitope within or including the RGS domain of p115 Rho-GEF is useful to detect the presence of the epitope in a sample, e.g., a sample of tissue containing p115 Rho-GEF gene product, distinguishing it from samples in which the epitope is absent.

Additionally, in accordance with the present invention, ligands which bind to an RGS domain of a GEF polypeptide can also be prepared, e.g., using synthetic peptide libraries or aptamers (e.g., Pitrung et al., U.S. Pat. No. 5,143,854; Geysen et al., 1987, J. Immunol. Methods, 102:259-274; Scott et al., 1990, Science, 249:386; Blackwell et al., 1990, Science, 250:1104; Tuerk et al., 1990, Science, 249: 505.

Antibodies and other ligands which bind the RGS domain of a GEF polypeptide, and specifically antibodies and other ligands which bind the RGS domain of p115 Rho GEF, can be used in various ways. These include, but are not limited to, uses therapeutic, diagnostic, and commercial research tools, e.g, to quantitate the levels of p115 Rho-GEF polypeptide in

animals, tissues, cells, etc., to identify the cellular localization and/or distribution of p115 Rho-GEF, to purify p115 Rho-GEF or a polypeptide comprising a part of p115 Rho-GEF, to modulate the function of p115 Rho-GEF, etc. Antibodies can be used in Western blots, ELIZA, immunoprecipitation, RIA, etc. The present invention relates to such assays, compositions and kits for performing them, etc.

An antibody according to the present invention can be used to detect polypeptides or fragments containing an RGS domain of a GEF polypeptide in various samples, including tissue, cells, body fluid, blood, urine, cerebrospinal fluid. A method of the present invention comprises contacting a ligand which binds to an RGS-GEF polypeptide of Figure 10, 12, 14, or 16 under conditions effective, as known in the art, to achieve binding, detecting specific binding between the ligand and peptide. By specific binding, it is meant that the ligand attaches to a defined sequence of amino acids, e.g., within or including the amino acid sequence of the RGS domain as shown in Figures 10, 12, 14, and 16, or derivatives thereof. The antibodies or derivatives thereof can also be used to inhibit expression of GEF proteins containing an RGS domain. The levels of a GEF polypeptide containing an RGS domain may be determined alone or in combination with other gene products. In particular, the amount (e.g., its expression level) of the GEF polypeptide containing an RGS domain can be compared (e.g., as a ratio) to the amounts of other polypeptides in the same or different sample, e.g., p21, p53, Rb, WT1, etc.

A ligand for the RGS domain of GEF polypeptides can be used in combination with other antibodies, e.g., antibodies that recognize oncological markers of cancer, including, Rb, p53, c-erbB-2, oncogene products, etc. In general, reagents which are specific for the RGS domain of GEF polypeptides can be used in diagnostic and/or forensic studies according to any desired method, e.g., as U.S. Pat. Nos. 5,397,712; 5,434,050; 5,429,947.

The present invention also relates to a transgenic animal, e.g., a non-human-mammal, such as a mouse, comprising an RGS-GEF polypeptide. Transgenic animals can be prepared according to known methods, including, e.g., by pronuclear injection of recombinant genes into pronuclei of 1-cell embryos, incorporating an artificial yeast chromosome into embryonic stem cells, gene targeting methods, embryonic stem cell methodology. See, e.g., U.S. Patent Nos. 4,736,866; 4,873,191; 4,873,316; 5,082,779; 5,304,489; 5,174,986; 5,175,384; 5,175,385; 5,221,778; Gordon et al., *Proc. Natl. Acad.*

Sci., 77:7380-7384 (1980); Palmiter et al., *Cell*, 41:343-345 (1985); Palmiter et al., *Ann. Rev. Genet.*, 20:465-499 (1986); Askew et al., *Mol. Cell. Bio.*, 13:4115-4124, 1993; Games et al. *Nature*, 373:523-527, 1995; Valancius and Smithies, *Mol. Cell. Bio.*, 11:1402-1408, 1991; Stacey et al., *Mol. Cell. Bio.*, 14:1009-1016, 1994; Hasty et al., *Nature*, 350:243-246, 1995; Rubinstein et al., *Nucl. Acid Res.*, 21:2613-2617,1993. A nucleic acid according to the present invention can be introduced into any non-human mammal, including a mouse (Hogan et al., 1986, in *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), pig (Hammer et al., *Nature*, 315:343-345, 1985), sheep (Hammer et al., *Nature*, 315:343-345, 1985), cattle, rat, or primate. See also, e.g., Church, 1987, *Trends in Biotech.* 5:13-19; Clark et al., 1987, *Trends in Biotech.* 5:20-24; and DePamphilis et al., 1988, *BioTechniques*, 6:662-680. Additionally, custom transgenic rat and mouse production is commercially available. These transgenic animals are useful, for example, as a cancer model or as a model to evaluate the effects of overexpression of the RGS-GEF polypeptide.

Generally, the nucleic acids, polypeptides, antibodies, etc. of the present invention can be prepared and used as described in, U.S. Pat. Nos. 5,501,969, 5,506,133, 5,441,870; WO 90/00607; WO 91/15582;

Other aspects of this invention relate to methods to assay for, or identify, molecules that modulate the following interactions and effects: the interaction between an RGS domain of a GEFand its cognate binding substrate; the interaction between an RGS domain of a GEF and a Gα subunit; the effect of G protein subunit stimulation on a guanine nucleotide exchange activity of a GEF protein containing an RGS domain; the effect of a GEF protein having an RGS domain as a GTPase activating protein for a G protein subunit.

Activity can be modulated in various ways, e.g., enhancing, activating, stimulating, suppressing, preventing, inhibiting, etc. A modulatory molecule can be an agonist, antagonist, or have partial activities thereof. Modulating molecules can be any type of molecule, including but not limited to small molecules, proteins, peptides, antibodies, nucleic acids, etc. In general, a compound having an *in vitro* activity will be useful *in vivo* to modulate a biological pathway associated with a GEF protein containing an RGS domain, e.g., to treat a pathological condition associated with the biological and cellular activities

mentioned above. The modulatory molecules can comprise a mixture of the same or different molecules.

A binding substrate for the RGS domain of a GEF protein can be any material to which the RGS domain binds specifically, including members of the Gα12 family. See, e.g., Strathman and Simon, *Proc. Natl. Acad. Sci.*, 88:5582, 1991. For example, a method of identifying or assaying for a molecule that modulates or regulates the binding of a G protein α subunit to a GEF protein containing an RGS domain, such as p115 Rho-GEF, can be conducted in accordance with this invention. In one embodiment, a GTP bound α subunit, or derivative thereof, is incubated with a GEF protein, or fragment thereof, containing the RGS domain, in the presence and absence of a test molecule to determine whether the presence of the test compound modulates the binding between the GEF protein and the G protein α subunit. The incubation is accomplished under effective conditions, i.e., conditions under which binding or attachment occurs. Binding can be detected in one or more ways. For example, the GEF protein or the binding substrate is labeled detectably; the labelled bound component is separated from the labelled free component; and the amount of bound-detectably labeled GEF protein or binding substrate determined. The detectable label can be of any desired composition, e.g., radioactive, fluorescent, etc. Such an assay can be performed in either solid or liquid phase.

In one aspect of the invention, it is desirable to identify molecules that regulate the binding of the $G\alpha_{12}$ family of subunits, eg. $G\alpha_{12}$ and $G\alpha_{13}$, with a GEF, e.g., p115 Rho GEF, Lsc, KIAA380, or DrhoGEF2. The assay can be conducted using a complete GEF protein, or any subfragments thereof which contain the RGS domain, or biologically active subfragments of the RGS domain. The assay is typically conducted with stable analogs of the GTP bound state of the Gα subunit, including α subunits bound to either GDP-AlF_4^- or GTPγS. For example, a binding assay may be conducted by the procedure described in Example 5 below wherein a COS cell is transfected with a nucleic acid construct for a myc-tagged polypeptide, such as p115 Rho GEF, or a fragment thereof and complexes of the polypetide and a Gα subunit are detected by precipitation of any bound complex with a first antibody to one of components and detection of the amount of a second bound component with a second antibody. Binding assays could also be performed using techniques that are

well known in the art such as by binding one of the components to a column and then determining the amount of a second labelled compoent that binds to the column. Relevant assay methods are also disclosed, for example, in Berman et al, 1996, *J. Biol. Chem.* 271:27209.

A method of isolating or assaying for a molecule that modules or regulates the stimulatory effect of a RGS-GEF polypeptide on GTPase activity, such as a GTPase activity of a Gα subunit, can also be conducted in accordance with the invention. For example, a Gα subunit is incubated under effective conditions with an RGS-GEF polypeptide having GTPase stimulatory effect in the presence and absence of a test inhibitor to determine whether the presence of the test inhibitor modulates its stimulatory effect. The assay can conducted using a complete RGS-GEF polypeptide, a GEF protein, or any subfragments thereof which contain the RGS domain, or biologically active subfragments of the RGS domain. An RGS-GEF polypeptide can be p115 Rho GEF, Lsc, KIAA380, DrhoGEF2, or biologically-active fragments thereof. For example, an assay can be conducted using a p115 Rho GEF in conjuction with an α12 or an α13 subunit, as described in the examples discussed herein, as well as using other variations or assay methods which are well known in the art. For example, the assay may be conducted in accordance with Example 5 below, in which Gα subunits were loaded with [γ-^{32}P]GTP and the amount of hydrolysis under various conditions, including the presence of an RGS-GEF polypeptide, was determined by measuring the amount of ^{32}Pi in the supernatant after centrifugation of the assay mixture. Relevant assay methods are also disclosed, for example, in Berman et al, 1996, *J. Biol. Chem.* 271:27209.

A method of identifying or assaying for a molecule that modulates the stimulatory effect of an activated Gα subunit on a RGS-GEF polypeptide having GEF mediated nucleotide exchange for a monomeric G protein can also be conducted in accordance with this invention. For instance, a first assay can be conducted by incubating an activated Gα alpha subunit with a GEF protein (e.g., p115 Rho GEF, Lsc, KIAA380, DrhoGEF2, or biologically-active fragments thereof, which retain GEF activity) and a monomeric G protein in the presence and absence of a test modulator to determine whether the test modulator has an inhibitory, enhancing, etc. effect on the ability of an activated Gα subunit

to stimulate GEF mediated nucleotide exchange of a monomeric protein. See e.g. Hart et al., 1996, *J. Biol. Chem.* 221:25452. The test modulator can be further evaluated by conducting a second assay in which said GEF protein and a monomeric G protein, without the G protein subunit, are incubated in the presence or absence of the test modulator to determine whether the test modulator had any effect on GEF mediated nucleotide exchange of the monomeric protein, and then comparing the modulation effect in the first and second assays to determine whether the modulating effect in the first assay is different from the modulating effect in the second assay, thereby indicating that the test modulator modulates the interaction of an activated Gα subunit with the GEF protein rather than the interaction of the GEF protein with the monomeric G protein. For example, the stimulatory effect on GEF mediated guanine nucleotide exchange may be measured according to Example 6 below, wherein RhoA was loaded with [^{3}H]GDP and the dissociation of GDP from RhoA was measured under various conditions by the determination of bound GDP by filtration, prior to an after incubation. (See e.g. Northrup et al., *J. Biol. Chem., 257*, 11416-11423 (1982)).

A method of identifying a molecule that mimics the stimulatory effect of an activated Gα subunit on GEF mediated nucleotide exchange of a monomeric G protein may also be conducted in accordance with the invention. The method comprises identifying a test compound that exhibits a binding affinity for the RGS domain of GEF proteins and then incubating a GEF protein and monomeric G protein in the presence or absence of the test compound to determine whether the test compounds exhibits a stimulatory effect on GEF mediated nucleotide exchange of a monomeric G protein. The identification of test compounds that exhibit a binding affinity for the RGS domain of GEF proteins may be accomplished using techniques well known in the art. For example, an RGS polypeptide may be bound to a column and cocktails of test compounds may be passed over the column to determine if any were selectively bound by the column.

A method of identifying a molecule, or mixture of molecules, that mimics the stimulatory effect of an RGS domain of GEF polypeptide on Gα subunit GTPase activity may also be conducted in accordance with the invention. The method comprises identifying a test compound that exhibits a binding affinity for a Gα subunit and incubating a GTP loaded Gα subunit in the presence or absence of the test compound to determine whether the test compound exhibits a stimulatory effect GTPase activity of the Gα subunit. The

identification of test compounds that exhibit a binding affinity for the G α subunit may be accomplished using techniques well known in the art. For example, a $G\alpha_{12}$ may be bound to a substrate and incubated with both a GEF polypeptide containing an RGS domain and the test compound to determine whether the test compound competes with the RGS domain for binding to the Gα subunit.

The modulation of oncogenic transforming activity by an RGS-GEF component, or derivatives thereof, can be measured according to various known procedures, e.g., Eva and Aaronson, Nature, 316:273-275, 1985; Hart et al., J. Biol. Chem., 269:62-65, 1994. A compound can be added at any time during the method (e.g., pretreatment of cells; after addition of the RGS-GEF, etc.) to determine its effect on the oncogenic transforming activity of the RGS-GEF component. Various cell lines can also be used.

Other assays for monomeric GTPase-mediated signal transduction can be accomplished according to the invention by analogy to procedures known in the art, e.g., as described in U.S. Pat. Nos. 5,141,851; 5,420,334; 5,436,128; and 5,482,954; WO94/16069; WO93/16179; WO91/15582; WO90/00607.

The present invention thus also relates to the treatment and prevention of diseases and pathological conditions associated with signal transduction mediated by GEF proteins that contain an RGS domain, e.g., cancer, diseases associated with abnormal cell proliferation. For example, the invention relates to a method of treating cancer comprising administering, to a subject in need of treatment, an amount of a compound effective to treat the disease, where the compound is a regulator of the stimulatory effect of GEF protein containing an RGS on Gα subunit GTPase activity or where the compound is a regulator of the stimulatory effect of a Gα subnit on GEF mediated nucleotide exchange by a monomeric GTPase. Treating the disease can mean, delaying its onset, delaying the progression of the disease, improving or delaying clinical and pathological signs of disease. A regulator compound, or mixture of compounds, can be synthetic, naturally-occurring, or a combination. A regulator compound can comprise amino acids, nucleotides, hydrocarbons, lipids, polysaccharides, etc. A regulator compound is preferably a compound that regulates expression of a GEF protein containing an RGS domain, e.g., inhibiting or increasing its mRNA, protein expression, or processing, or a compound that regulates the interaction of the RGS domain of the GEF protein with a Gα subunit. To treat the disease, the compound,

or mixture, can be formulated into pharmaceutical composition comprising a pharmaceutically acceptable carrier and other excipients as apparent to the skilled worker. See, e.g., *Remington's Pharmaceutical Sciences*, Eighteenth Edition, Mack Publishing Company, 1990. Such composition can additionally contain effective amounts of other compounds, especially for treatment of cancer.

EXAMPLES

Example 1. Identification of homology between a Rho GEF and proteins which regulate G-protein signaling.

The RGS family of proteins act as negative regulators of G protein signalling. Nineteen mammalian members of the family have been identified, all of which encode proteins that contain a homologous core domain called the RGS box.

Examination of the sequence of p115-GEF, a GEF specific for Rho, revealed an N-terminal region with specific homology to the conserved domain of RGS proteins, including RGS4, RGS2, GAIP, RGS12, and RGS14 (Fig. 1). Analysis of three other Rho GEF proteins, Lsc, KIAA380, and DrhoGEF also showed that they contained regions of specific homology to the conserved domain of RGS proteins (Fig. 1).

The crystal structure of a complex between RGS4 and AlF_4-activated $G\alpha_{i1}$ revealed that the functional core of RGS4 (the RGS box) contains nine α-helixes that fold into two small subdomains (Tesmer et al., *Cell*, **89**, 251 (1997)). The RGS box has been shown to contain the GAP activity towards Gα subunits (Popov et al., *Proc. Natl. Acad. Sci.* USA, **94**, 7216 (1997)). The hydrophobic core residues of the box, which are conserved in members of the RGS family, are important for stability of structure and GAP activity (Tesmer et al., *Cell*, **89**, 251 (1997) and Srinivasan et al., *J. Biol. Chem.*, **273**, 1529 (1998). RGS4 stimulates the GTPase activity of $G\alpha_{i1}$ by interacting with its three switch regions, primarily by stabilization of the transition state of GTP hydrolysis (Tesmer et al., *Cell*, **89**, 251 (1997)).

Most of the hydrophobic residues that form the core of the RGS domain are conserved in p115 Rho GEF (17 out of 23) (Fig. 1). The position of gaps in the alignment correspond to the loops between alpha helixes of RGS domain structure. This homology suggested that the N-terminal region of p115-GEF may have a similar structure to the RGS4 box domain and possess GAP activity. In contrast, the residues of RGS4 that make contact

with the switch regions of $G\alpha_{i1}(GDP\text{-}AlF_4\text{-})$ are not well conserved, and any GAP activity of p115 Rho GEF will have a unique mechanism or a significantly different specificity than those previously identified.

A search of the gene bank revealed three other Rho-GEF members that have regions homologous to the RGS region of p115. These include Lsc, KIAA380, and DrhoGEF2 (Fig. 1). Lsc appears to be the mouse homolgue of p115 RhO GEF and KIAA380 appears to be the human homolgue of Drosophila DrhoGEF2 (Whitehead et al., *J. Biol. Chem.*, **271**, 18643 (1996); Barrett et al., *Cell*, **91**, 905 (1997)). These four Rho-GEF's define a new RGS related family of proteins which also possess guanine nucleotide exchange activtity for Rho.

An alignment of RGS domains of the four GEF proteins known to contain RGS domains (p115 Rho GEF, Lsc, KIAA380, DRhoGEF2) with the RGS domains of RGS proteins RET-RGS1, RGS1, RGS2, RGS3, RGS4, RGS7, RGS10, RGS12, RGS14, Rap1/2B.P., and GAIP shows that a novel sub-RGS consensus sequence is defined by the RGS sequence of the four GEF proteins (Fig. 18). As shown in the bottom set of sequences shown in Fig. 18, a novel sub-RGS consensus sequence is shown by the large gap of 13 to 14 amino acids in the homology alignment, along with the conservation of amino acids on either side of the gap.

Example 2. <u>The RHO GEF protein, p115 RHO-GEF, stimulates the GTPase activity of $G\alpha_{13}$ and $G\alpha_{12}$ subunits</u>.

P115 Rho GEF was tested to determine it's capability in stimulating the intrinsic GTPase activity (GAP activity) of $G\alpha_{13}$ and $G\alpha_{12}$.

$G\alpha_{12}$ was expressed in Sf9 cells and purified as described in Kozasa and Gilman, *J. Biol. Chem.*, **270**, 1734 (1995). $G\alpha_{13}$ was prepared by a similar procedure using the previously described baculovirus method (Singer and Miller, *J. Biol. Chem.*, **269**, 19796 (1994)) and octylglucoside during washing and elution of the α subunit after immobilization of the heterotrimer on Ni-NTA resin (Qiagen). The eluted $G\alpha_{13}$ was further purified by absorption to and elution from hydroxyapatite. $G\alpha_{12}$ or $G\alpha_{13}$ (20-30 pmol) was loaded at 30°C for 30 or 40 minutes, respectively with 5 $\mu M[\gamma\text{-}^{32}P]GTP$ (50-100 cpm/fmol) and in the

presence of 5 mM EDTA. Samples were then rapidly filtered by centrifugation at 4°C through Sephadex G50 which had been equilibrated with buffer A (50 mM NaHepes (pH 8.0), 1 mM dithiolthreitol, 5 mM EDTA, and 0.05% polyoxyethylene 10-laurylether) to remove free [γ-^{32}P]GTP and [^{32}Pi]. Hydrolysis of GTP was initiated by adding Gα loaded with [γ-^{32}P]GTP in buffer A containing 8 mM $MgSO_4$, 1 mM GTP and the indicated amount of p115. The reaction mixture was incubated at 4°C or 15°C. Aliquots (50 µl) were removed at the indicated times and mixed with 750 µl of 5%(w/v) NoritA in 50 mM NaH_2PO_4. The mixture was centrifuged at 2000 rpm for 5 minutes and 400 µl of supernatant containing ^{32}Pi were counted by liquid scintillation spectrometry.

The hydrolysis of GTP bound to Gα_{13} and Gα_{12} was performed at 15°C either with or without 10nM full-length p115 (Fig. 2, Panel A). The hydrolysis of GTP bound Gα_{13} and Gα_{12} was measured at 4°C in the presence of various concentrations of p115 (Fig. 2, Panel B). Full-length p115 was able to stimulate a single round of hydrolysis of [γ-^{32}P]GTP which had been prebound to the Gα_{13} subunit. The intrinsic GTPase activity of Gα_{12}, the closest homologue of Gα_{13}, was also stimulated by full-length p115. At 15°C, the k_{cat} for hydrolysis of GTP by Gα_{12} (0.07 min^{-1}) and Gα_{13} (0.24 min^{-1}) were respectively increased 5-fold and 10-fold by 10 nM p115 (Fig. 2, Panel A). Similar results were obtained with several preparations of Gα_{12} and Gα_{13}. Treatment of p115 at 90°C inactivated this GAP activity. Due to the rapid hydrolytic rates of Gα_{13}, assays were performed at 4°C to better estimate the effect of p115 on the initial rate of GTPase activity by the G protein (Fig. 2, Panel B). Under these conditions, 100 nM p115 caused and 80-fold increase in the GTPase activity of Gα_{13}. In contrast, the hydrolytic rate of Gα_{12} was increased only 6-fold. Although stimulation of both proteins was observed at concentrations of p115 as low as 1 nM, measurements at both temperatures indicate that p115 is a more efficacious GAP for Gα_{13} than Gα_{12}.

In the absence of a receptor, the rate limiting step in the binding of GTPγS to Gα and the steady state hydrolysis of GTP is the release of GDP. P115 did not affect either the rate of GTPγS binding to Gα_{12} and Gα_{13} or the steady state of GTPase activity of either subunit.

Therefore, p115 stimulates only the intrinsic GTPase activity of $G\alpha_{12}$ and $G\alpha_{13}$ without effecting their rates of nucleotide exchange.

The conserved RGS box region of RGS proteins is sufficient to show GAP activity *in vitro* (Popov et al., *Proc. Natl. Acad. Sci.* USA, **94**, 7216 (1997)). Therefore, a fusion protein (Fig. 1, Panel B) of glutathione-S-transferase and the N-terminal region of p115, GST-RGS, was tested for GAP activity. This region retains RGS homology domain but not the Dbl or PH domains of p115. This "RGS domain" of p115 (10 nM) was almost as active as full-length p115 when tested for GAP activity for $G\alpha_{12}$ and $G\alpha_{13}$ (Fig. 3). In contrast, a construct of p115 missing this N-terminal region was ineffective. Thus, the data indicates that the RGS homology region is responsible for the GAP activity of p115.

Example 3. The p115 RHO-GEF, does not stimulate the GTPase activity of $G\alpha_i$, $G\alpha_z$ $G\alpha_q$ and $G\alpha_s$ subunits.

The specificity of the GAP activity of p115 for various G protein α subunits was examined as follows.

$G\alpha_S$ was expressed in and purified from *Escherichia coli* as described in Lee et al., *Meth. Enzymol.*, **237**, 146 (1994). $G\alpha_i$, $G\alpha_z$, and $G\alpha_q$R183C were expressed in Sf9 cells and purified as described in Kozasa and Gilman, *J. Biol. Chem.*, **270**, 1734 (1995) and Biddlecome et al., *J. Biol. Chem.*, **271**, 7999 (1996). $G\alpha_i$, $G\alpha_z$, and $G\alpha_z$ were loaded with 5-10 μM [γ-^{32}P]GTP at 20°C (for $G\alpha_s$) or 30°C (for $G\alpha_i$ and $G\alpha_z$) for 20 minutes in the presence of 5 mM EDTA and GAP assays were performed as described above for $G\alpha_{12}$ and $G\alpha_{13}$. Gap activity on $G\alpha_q$ was assessed with a mutant $G\alpha_q$R183C. An analogous mutation in $G\alpha_i$ R1178C, causes markedly reduced GTPase activity but response to RGS proteins was retained (Berman et al., *Cell*, **86**, 445 (1996)). The slow GTPase activity of $G\alpha_q$R183C enables loading of [γ-^{32}P]GTP on $G\alpha_q$ without using receptor to accelerate nucleotide exchange. $G\alpha_q$R183C was loaded with 10 μM [γ-^{32}P]GTP in the presence of 50 mM Hepes (pH7.4), 0.1 mg/ml BSA, 1 mM DTT, 1 mM EDTA, 0.9 mM $MgSO_4$, 30 mM $(NH_4)_2SO_4$, 4% glycerol, and 5.5 mM CHAPS at 20°C for 2 hours. The reaction mixture was rapidly

filtered through Sephadex G50 which had been equilibrated with 50 mM Hepes (pH 7.4), 1 mM DTT, 1 mM EDTA, 0.9 mM SO_4, 0.1 mg/ml BSA, and 1 mM CHAPS.

The results of this study showed that p115 (100 nM) did not stimulate the GTPase activity of $G\alpha_i$, $G\alpha_z$, or $G\alpha_q$ under conditions where RGS4 acts as a GAP for these $G\alpha$ subunits (Figure 4). Similarly, p115 did not accelerate the GTPase activity of $G\alpha_s$, nor did p115 Rho GEF have any GAP activity towards RhoA or rac1. Thus, p115 is a GAP with specificity for $G\alpha_{12}$ and $G\alpha_{13}$.

Example 4. Selective inhibition of p115 GAP activity by AlF_4^- activated forms of $G\alpha$ subunits.

RGS proteins have been shown to have high affinity for the GDP-AlF_4^- bound form of α subunits, a configuration similar to the transition state of GTP hydrolysis (Tesmer et al., *Cell*, **89**, 251 (1997), Berman et al., *J. Biol. Chem.*, **271**, 27209 (1996)). Therefore, the GDP-AlF_4^- forms of $G\alpha$ should compete with $G\alpha$GTP for interaction with p115 and block the observed GAP activity. As shown in Fig. 5, Panel A, GDP-AlF_4^- bound $G\alpha_{12}$ and $G\alpha_{13}$ effectively inhibited the GAP activity of p115 for $G\alpha_{12}$, while similar forms of $G\alpha_s$, $G\alpha_i$, and $G\alpha_q$ were without effect. Additionally, a tritration of GDP-AlF_4^- bound forms of $G\alpha_{12}$ and $G\alpha_{13}$ demonstrated that the subunits are equipotent in inhibiting the GAP activity of $G\alpha_{13}$ (Fig. 5, Panel B). These competition assays suggest that the two G protein subunits have a similar affinity for p115 and supports the apparent differential efficacy of p115 towards the subunits as shown in Fig. 2.

Example 5. Binding of $G\alpha_{13}$ to p115 Rho GEF in vivo.

The following experiments demonstrated that $G\alpha_{13}$ and p115 Rho GEF interact in a GTP-dependent manner.

EXV-myc tagged (for COS cell transfections) and pAc-Glu tagged (for baculovirus expression) proteins with deletions of the RGS or DH domains were constructed as previously described in Hart et al., *J. Biol. Chem.*, **271**, 25452-25458 (1996). Full-length versions were constructed in the same vectors. A fusion of GST to the first 246 amino acids of p115 Rho GEF was constructed in pGEX4T-2 (Pharmacia). Transfections,

immunoprecipitations, and purifications were performed as previously described in Hart et al., *J. Biol. Chem.*, **271**, 25452-25458 (1996).

In COS cells transfected with myc-tagged p115 Rho GEF, $G\alpha_{13}$ can be specifically immunoprecipitated using the anti-myc antibody (Fig. 6, Panels A and B). This interaction is dependent on the presence of aluminum fluoride which is added to mimic the activated GTP-bound state of the $G\alpha_{13}$. Additionally, a truncated mutant of p115 Rho GEF which lacks the amino-terminal RGS domain is incapable of mediating co-immunoprecipitation, while full-length protein with a deletion in the DH domain does mediate co-immunoprecipitation. The differential binding of full-length and truncated Rho GEF proteins could also be detected using antibodies to $G\alpha_{13}$ to immunoprecipitate the complex (Fig. 6, Panel C). A very weak interaction with $G\alpha_{12}$ was detectable, while antibodies to $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_z$ do not detect immunoreactive bands in the anti-myc immunoprecipitates, in spite of the fact that their respective antigens are detectable in the whole cell lysates. The co-immunoprecipitation of p115 Rho GEF and $G\alpha_{13}$ can be reproduced in a semi-purified system in which purified $G\alpha_{13}$ is added to immunoprecipitated p115 Rho GEF (Fig. 6, Panel D), suggesting a direct interaction. This direct interaction is consistent with the observation that p115 Rho GEF stimulates $G\alpha_{13}$ GTPase activity, but also indicates that p115 Rho may be an effector of $G\alpha_{13}$.

Binding could also be detected between the Rho GEF protein, KIAA380 and the α_{12} G protein subunit (Fig. 9, KIAA380 is referred to as FL147). In COS cells transfected with myc-tagged KIAA380, $G\alpha_{12}$ can be specifically immunoprecipitated using the anti-myc antibody (Fig. 9, Panels A and B, KIAA380 is referred to as FL147). This interaction is dependent on the presence of aluminum fluoride which is added to mimic the activated GTP-bound state of the $G\alpha_{13}$.

Example 6. Stimulation of p115 Rho GEF activity by $G\alpha_{13}$.

The ability of $G\alpha_{13}$ to affect the exchange activity of p115 Rho GEF was examined by incubating RhoA and p115 Rho GEF with or without $G\alpha_{13}$ to determine the effect on guanine nucleotide exchange.

RhoA (2.5 μM) was loaded with [^{3}H]GDP by incubation at 30°C for 1 hour with 25 μM GDP (10,000 cpm/pmol) in 50 mM NaHepes, pH 7.5, 50 mM NaCl, 4 mM EDTA, 1mM dithiolthreitol and 0.1%Triton X-100. After addition of $MgCl_2$ to 9 mM and octylglucoside to 1%, the Rho was incubated for an additional 5 minutes and separated from free GDP by rapid filtration through Sephadex-G50 that had been equilibrated with 50 mM NaHEPES, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiolthreitol, 5 mM $MgCl_2$, and 1% octylglucoside. Dissociation of GDP from RhoA was measured at 30°C in 20 μl of 50 mM NaHEPES, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiolthreitol, 5 mM $MgCl_2$, 30 mM $AlCl_3$, 5 mM NaF, and 5 μM GTPγS. Unless specified, G protein alpha subunits were preincubated with AMF (30 μM $AlCl_3$, 5 mM $MgCl_2$ and 5 mM NaF) prior to mixing with other proteins. Where indicated, alpha subunits were treated with 25 μM GTPγS or GDPβS rather than AMF and reactions were incubated without AMF but with 5 μM of the respective nucleotide. Reactions were started with the addition of [^{3}H]-GDP-RhoA and bound GDP was determined by filtration (Northup et al., *J. Biol. Chem.*, **257**, 11416-11423 (1982)) prior to and after incubation.

The $G\alpha_s$ and $G\alpha_i$ alpha subunits were purified after expression in *Escherichia coli* (Lee et al., *Meth. Enzymol.*, **237**, 146-164 (1994)). The $G\alpha_q$ and $G\alpha_z$ alpha subunits were coexpressed in Sf9 cells with hexahistidine-tagged beta and gamma subunits and isolated as described (Kozasa and Gilman, *J. Biol. Chem.*, **270**, 1734-1741 (1995)). $G\alpha_{13}$ was prepared by a similar procedure to $G\alpha_{12}$ using baculovirus (Singer et al., *J. Biol. Chem.*, **269**, 19796-19802 (1994)) and octylglucoside during washing and elution of the α subunit after immobilization of the heterotrimer on Ni-NTA resin (Qiagen). The eluted $G\alpha_{13}$ was further purified by absorption to and elution from hydroxyapatite. About 500 ug of purified $G\alpha_{13}$ can be obtained from 3 liters of cells. The expression of GST-RhoA in Sf9 cells, cleavage of the GST tag and isolation of the free RhoA were as described in Singer et al., *J. Biol. Chem.*, **271**, 4505-4510, (1996).

These studies demonstrated that the $G\alpha_{13}$ is capable of stimulating the activity of full-length p115 Rho GEF in a manner which depends on the concentrations of both p115 Rho GEF (Fig. 7, Panel A) and $G\alpha_{13}$ (Fig. 7, Panel B). The closely related alpha subunit

$G\alpha_{12}$ was ineffective in stimulating the activity of p115 Rho GEF in these experiments (Fig. 7, Panel A). Stimulation of Rho exchange was also monitored as a function of the activation state of $G\alpha_{13}$. The data graphed in Fig. 7, Panel C confirm that the stimulation of exchange activity is dependent on either aluminum fluoride (AMF) or GTPγS, but is not stimulated by the deactivated nucleotide state mimicked by GDPβS. Additionally, a series of other alpha subunits including $G\alpha_q$, $G\alpha_z$, $G\alpha_s$, and $G\alpha_i$ also did not affect the activity of p115 Rho GEF (Fig. 7, Panel D). These results are consistent with with the activated $G\alpha_{13}$ -dependent binding shown in Figure 6, and suggest that the productive binding of $G\alpha_{13}$ to p115 Rho GEF may be sufficient for activation.

Example 7. Effects of domains of p115 and $G\alpha_{12}$ on the p115 nucleotide exchange activity.

The theory that the RGS domain of p115 Rho GEF is normally autoinhibitory and that binding to $G\alpha_{13}$ relieves this inhibition was examined by comparing the effects of full-length Rho-GEF versus truncated Rho-GEF on Rho exchange activity.

Preparation of p115 proteins was as described in Example 1 above and as described in Hart et al., *J. Biol. Chem.*, **271**, 25452-25458 (1996). The assays shown in Figure 8, Panels B and C were performed as described in Example 5 above. AMF was the activating agent.

The results of these experiments showed that truncated p115 Rho GEF lacking the RGS domain demonstrates consistently elevated Rho exchange activity when compared with equal concentrations of the full-length protein (Fig. 8, Panel A). Additionally, addition of the isolated RGS domain (as a GST fusion protein) resulted in abrogation of $G\alpha_{13}$-stimulated p115 Rho GEF activity (Fig. 8, Panel B). These data do not preclude additional $G\alpha_{13}$-binding sites on p115 Rho GEF, although they do suggest a primary mode of action via the RGS domain.

The inability of the $G\alpha_{12}$ subunit to activate p115 Rho GEF was puzzling in light of the fact that p115 Rho GEF is capable of activating the GTPase of both $G\alpha_{12}$ and $G\alpha_{13}$. Therefore, an experiment was conducted in which $G\alpha_{12}$ was added to a $G\alpha_{13}$-stimulated p115 Rho GEF assay (Fig. 8, Panel C). The results showed that $G\alpha_{12}$ was able to inhibit the

coupling of $G\alpha_{13}$ with p115 Rho GEF. This data is consistent with a model in which $G\alpha_{12}$ competes with $G\alpha_{13}$ for binding to the RGS domain of p115 Rho GEF. However, binding of $G\alpha_{12}$ to p115 Rho GEF is clearly not sufficient to stimulate Rho exchange activity. These results suggest that either the interaction of $G\alpha_{12}$ with the RGS domain of p115 Rho GEF is quite different from that of $G\alpha_{13}$ or that there may be an additional site of interaction between $G\alpha_{13}$ and p115 Rho GEF.

For other aspects of the nucleic acids, polypeptides, antibodies, etc., reference is made to standard textbooks of molecular biology, protein science, and immunology. See, e.g., Davis et al. (1986), *Basic Methods in Molecular Biology*, Elsevir Sciences Publishing, Inc., New York; Hames et al. (1985), *Nucleic Acid Hybridization*, IL Press, *Molecular Cloning*, Sambrook et al.; *Current Protocols in Molecular Biology*, Edited by F.M. Ausubel et al., John Wiley & Sons, Inc; *Current Protocols in Human Genetics*, Edited by Nicholas C. Dracopoli et al., John Wiley & Sons, Inc.; *Current Protocols in Protein Science*; Edited by John E. Coligan et al., John Wiley & Sons, Inc.; *Current Protocols in Immunology*; Edited by John E. Coligan et al., John Wiley & Sons, Inc. The entire disclosure of all patent applications, patents, and publications cited herein are hereby incorporated by reference.

From the foregoing description, on skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

CLAIMS

What is claimed is:

1. An isolated RGS-GEF polypeptide, or a biologically active fragment thereof, consisting essentially of an RGS domain of a GEF protein.

2. An isolated RGS-GEF polypeptide, or a biologically active fragment thereof, comprising an RGS domain of a GEF protein, with the proviso that the polypeptide does not comprise a DH domain or a PH domain.

3. An isolated RGS-GEF polypeptide, or a biologically active fragment thereof, wherein the polypeptide is selected from the group consisting of p115 Rho-GEF, Lsc, KIAA380, and wherein the polypeptide is mutated in the RGS domain, and wherein the polypeptide has a specific binding affinity for a G protein α subunit or a GTPase activating activity for a G protein α subunit.

4. An isolated RGS-GEF polypeptide, or a biologically active fragment thereof, according to claim 1 or 2, wherein the GEF protein is a Rho GEF protein.

5. An isolated RGS-GEF polypetide, or a biologically active fragment thereof, according to claim 4, wherein the Rho GEF protein is p115 Rho-GEF.

6. An isolated RGS-GEF polypeptide, or biologically active fragment thereof, according to claim 4 wherein the Rho GEF protein is selected from the group consisting of Lsc, KIAA380, and DrhoGEF2.

7. An isolated RGS-GEF polypeptide, or a biologically active fragment thereof , according to claim 1 or 2, wherein the polypeptide has a specific binding affinity for a G protein α subunit or a GTPase activating activity for G protein α subunits.

8. An isolated RGS-GEF polypeptide, or a biologically active fragment thereof, according to claim 4, wherein the polypeptide has a specific binding affinity for a G protein α subunit or a GTPase activating activity for G protein α subunits.

9. An isolated RGS-GEF polypeptide, or a biologically active fragment thereof, according to claim 5, wherein the polypeptide has a specific binding affinity for a G protein α subunit or a GTPase activating activity for G protein α subunits.

10. An isolated RGS-GEF nucleic acid consisting essentially of a nucleotide sequence encoding a polypeptide comprising an RGS domain of a GEF protein.

11. An isolated RGS-GEF nucleic acid comprising a nucleotide sequence encoding a polypeptide comprising an RGS domain of a GEF protein, wherein the polypeptide does not include a DH domain or a PH domain.

12. An isolated RGS-GEF nucleic acid according to claim 10 or 11, wherein the GEF protein is a Rho GEF protein.

13. An isolated RGS-GEF nucleic acid according to claim 12, wherein the Rho GEF protein is p115 Rho GEF.

14. An isolated RGS-GEF nucleic acid according to claim 12 wherein the Rho GEF protein is selected from the group consisting of Lsc, KIAA380, and DrhoGEF2.

15. An isolated RGS-GEF nucleic acid according to claim 10 or 11, wherein the polypeptide has a specific binding affinity for a G protein α subunit or a GTPase activating activity for a G protein α subunit.

16. An isolated RGS-GEF nucleic acid according to claim 12, wherein the polypeptide has a specific binding affinity for a G protein α subunit or a GTPase activating activity for a G protein α subunit.

17. An isolated RGS-GEF nucleic acid according to claim 13, wherein the polypeptide has a specific binding affinity for a G protein α subunit or a GTPase activating activity for a G protein α subunit.

18. A method of modulating an activity of a G protein α subunit comprising, administering to a mammal an effective amount of a polypeptide according to claim 1 or 4.

19. A method of identifying or assaying a molecule that inhibits or enhances binding of a monomeric G protein guanine nucleotide exchange factor to a G protein α subunit comprising incubating the G protein α alpha subunit, or fragments thereof, with the monomeric G protein nucleotide exchange factor, or fragments thereof, in the presence and absence of a test molecule and determining whether the presence of the test molecule inhibits or enhances binding between the monomeric G-protein guanine nucleotide exchange factor and the G protein α subunit.

20. A method of identifying or assaying a molecule that inhibits or enhances a stimulatory effect of a GEF on a Gα subunit GTPase activity comprising incubating a Gα alpha subunit, or fragments thereof, with a GEF protein, or fragments thereof, in the presence and absence of a test molecule and determining whether the presence of the test molecule inhibits or enhances the stimulatory effect of the GEF protein on Gα subunit GTPase activity.

21. A method of identifying or assaying a molecule that specifically inhibits the stimulatory effect of an activated Gα subunit on GEF mediated nucleotide exchange of a monomeric G protein, compising conducting a first assay by incubating an activated Gα alpha subunit, or fragments thereof, with a GEF protein, or fragments thereof, and a monomeric G protein, or fragments thereof, in the presence and absence of a test inhibitor, conducting a second assay by incubating a GEF protein,

or fragments thereof, and a monomeric G protein, or fragments thereof, in the presence and absence of the test inhibitor, and determining whether any inhibitory effect of the test inhibitor in the first assay is greater than any inhibitory effect of the test inhibitor in the second assay.

22. A method of identifying or assaying a molecule that specifically enhances the stimulatory effect of an activated Gα subunit on GEF mediated nucleotide exchange of a monomeric G protein, compising conducting a first assay by incubating an activated Gα alpha subunit, or fragments thereof, with a GEF protein, and fragments thereof, and a monomeric G protein, or fragments thereof, in the presence and absence of a test enhancer, conducting a second assay by incubating a GEF protein, or fragments thereof, and a monomeric G protein, or fragments thereof, in the presence and absence of the test enhancer, and determining whether any enhancing effect of the test enhancer in the first assay is greater than any enhancing effect of the test enhancer in the second assay.

23. A method of identifying or assaying a molecule that mimics the stimulatory effect of an activated Gα subunit on GEF mediated nucleotide exchange of a monomeric G protein comprising identifying a test compound that exhibits a binding affinity for the RGS domain of GEF proteins, or fragments thereof, incubating a GEF protein,or fragments thereof, and monomeric G protein, or fragments thereof, in the presence or absence of the test compound, determining whether the test compound exhibits a stimulatory effect on GEF mediated nucleotide exchange of a monomeric G protein.

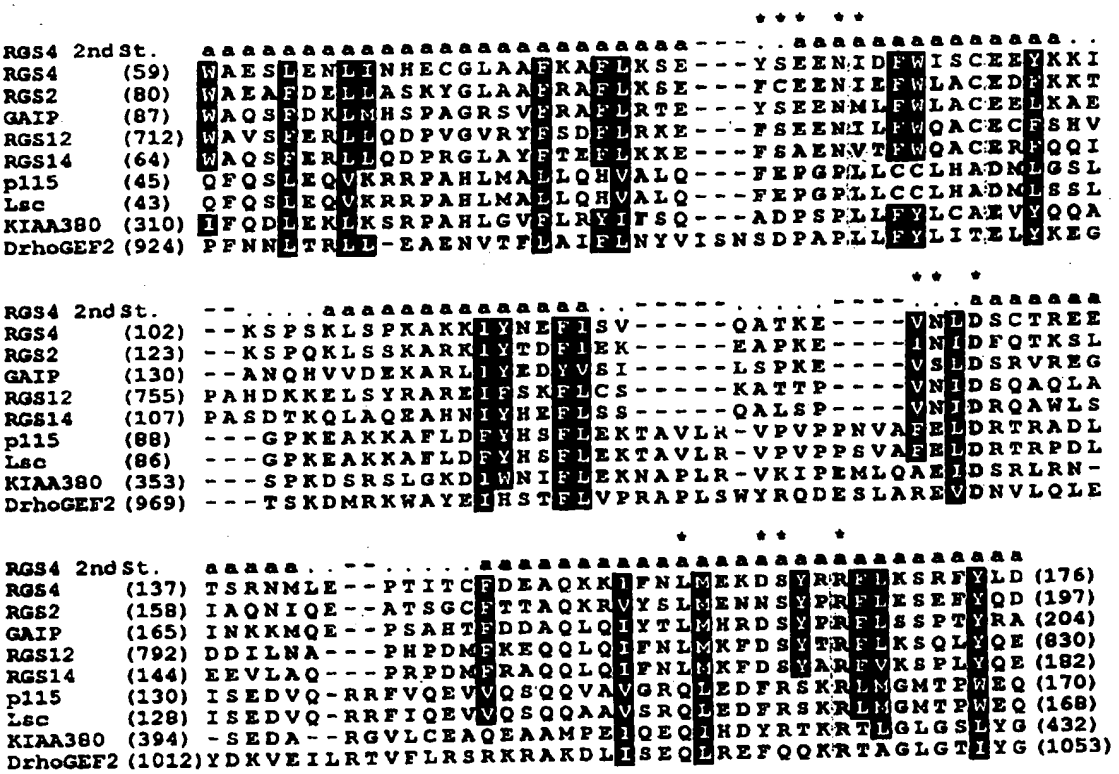
24. A method of identifying or assaying a molecule that mimics the stimulatory effect of an RGS domain of a GEF protein on GTPase activity of a Gα subunit comprising identifying a test compound that exhibits a binding affinity for a Gα subunit and incubating a GTP loaded Gα subunit in the presence or absence of the test compound to determine whether the test compound has a stimulatory effet on Gα subunit GTPase activity.

25. A method according to claim 19, 20, 21, 22, 23, or 24 wherein the GEF protein is selected from the group consisting of p115 Rho GEF, Lsc, KIAA380, and DrhoGEF2.

26. A method of expressing in transformed host cells, a polypeptide coded for by a nucleic acid, comprising culturing transformed host cells containing a nucleic acid according to claim 11.

27. A transformed cell containing a nucleic acid according to claim 11.

28. A vector comprising a nucleic acid according to claim 11.


RGS4 2nd St.
RGS4 (59)
RGS2 (80)
GAIP (87)
RGS12 (712)
RGS14 (64)
p115 (45)
Lsc (43)
KIAA380 (310)
DrhoGEF2 (924)
RGS4 (102)
RGS2 (123)
GAIP (130)
RGS12 (755)
RGS14 (107)
p115 (88)
Lsc (86)
KIAA380 (353)
DrhoGEF2 (969)
RGS4 (137) (176)
RGS2 (158) (197)
GAIP (165) (204)
RGS12 (792) (830)
RGS14 (144) (182)
p115 (130) (170)
Lsc (128) (168)
KIAA380 (394) (432)
DrhoGEF2 (1012) (1053)

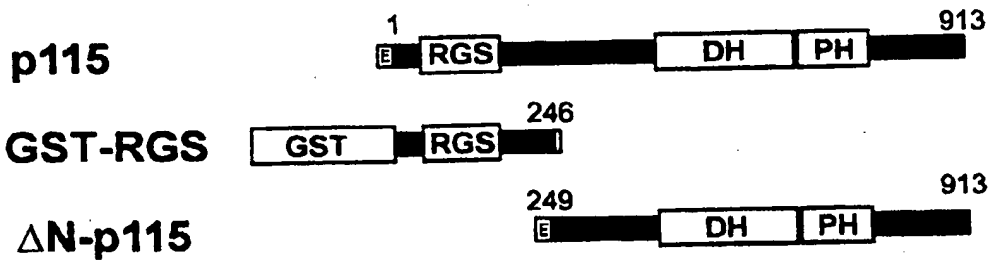

p115
1
913
RGS
DH
PH
GST-RGS
GST
RGS
246
ΔN-p115
249
913
DH
PH

FIGURE 1

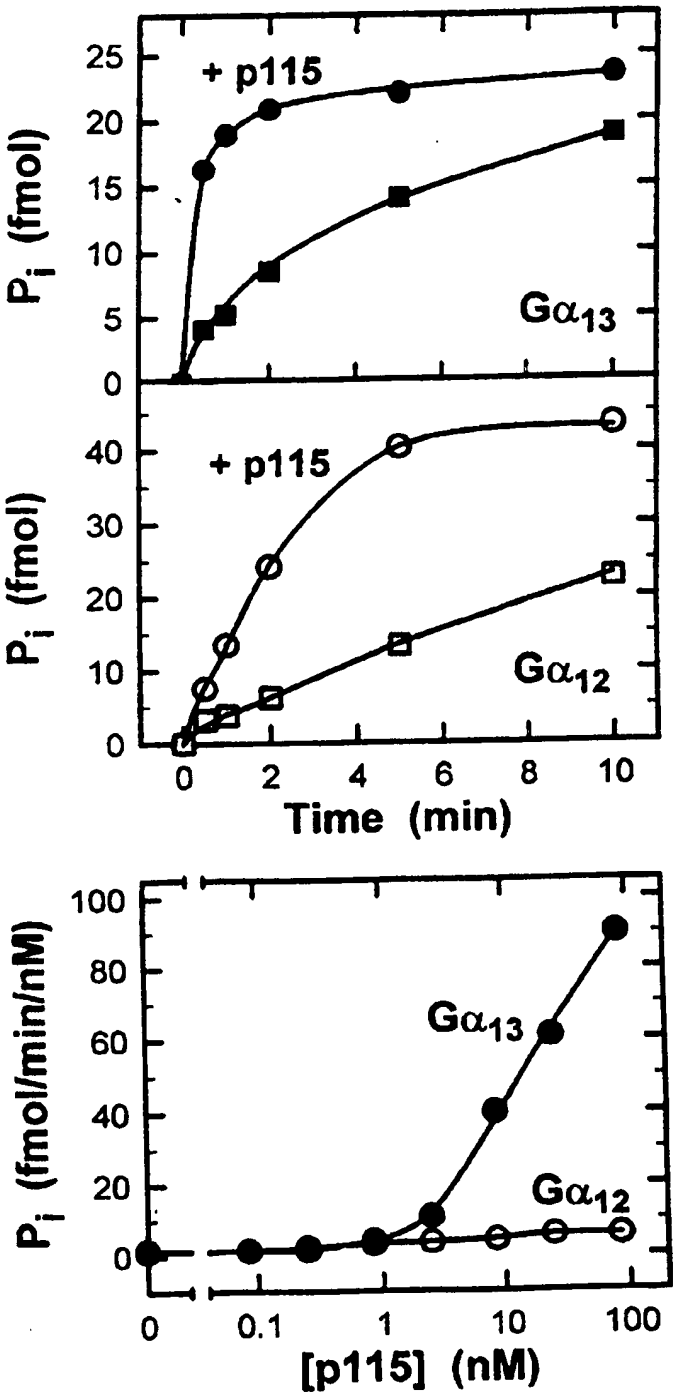

+ p115
Gα13
+ p115
Gα12
Pi (fmol)
Time (min)
Gα13
Gα12
Pi (fmol/min/nM)
[p115] (nM)

FIGURE 2

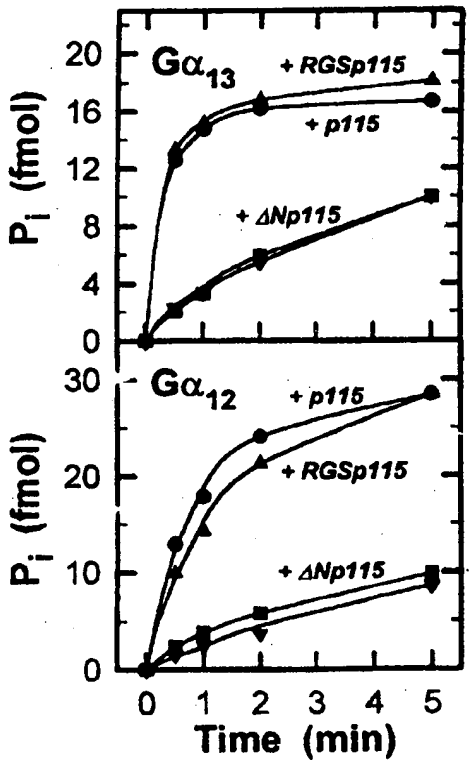

Gα13
+ RGSp115
+ p115
+ ΔNp115
Pi (fmol)
20
16
12
8
4
0
Gα12
+ p115
+ RGSp115
+ ΔNp115
Pi (fmol)
30
20
10
0
0 1 2 3 4 5
Time (min)

FIGURE 3

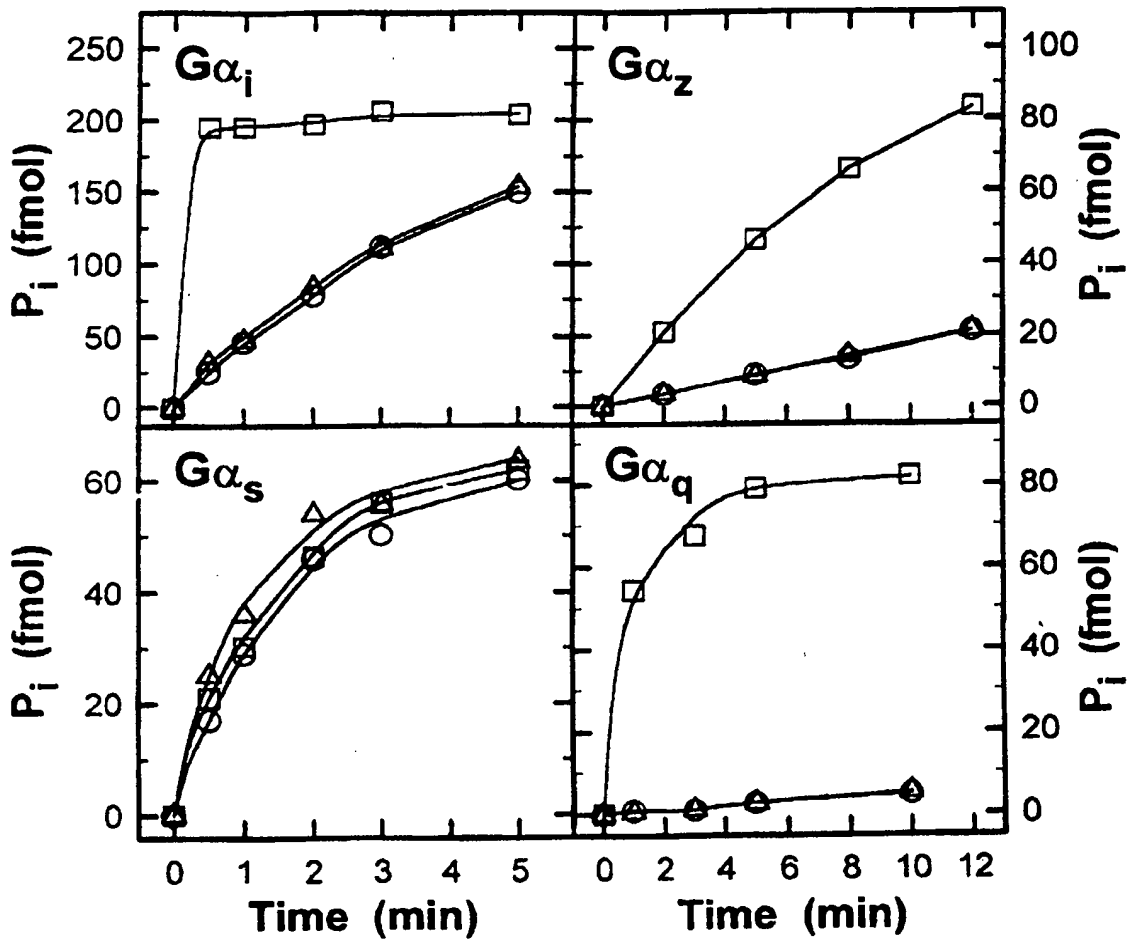

Gαi
Gαz
Gαs
Gαq
Pi (fmol)
Time (min)
0
50
100
150
200
250
20
40
60
80
1
2
3
4
5
6
8
10
12

FIGURE 4

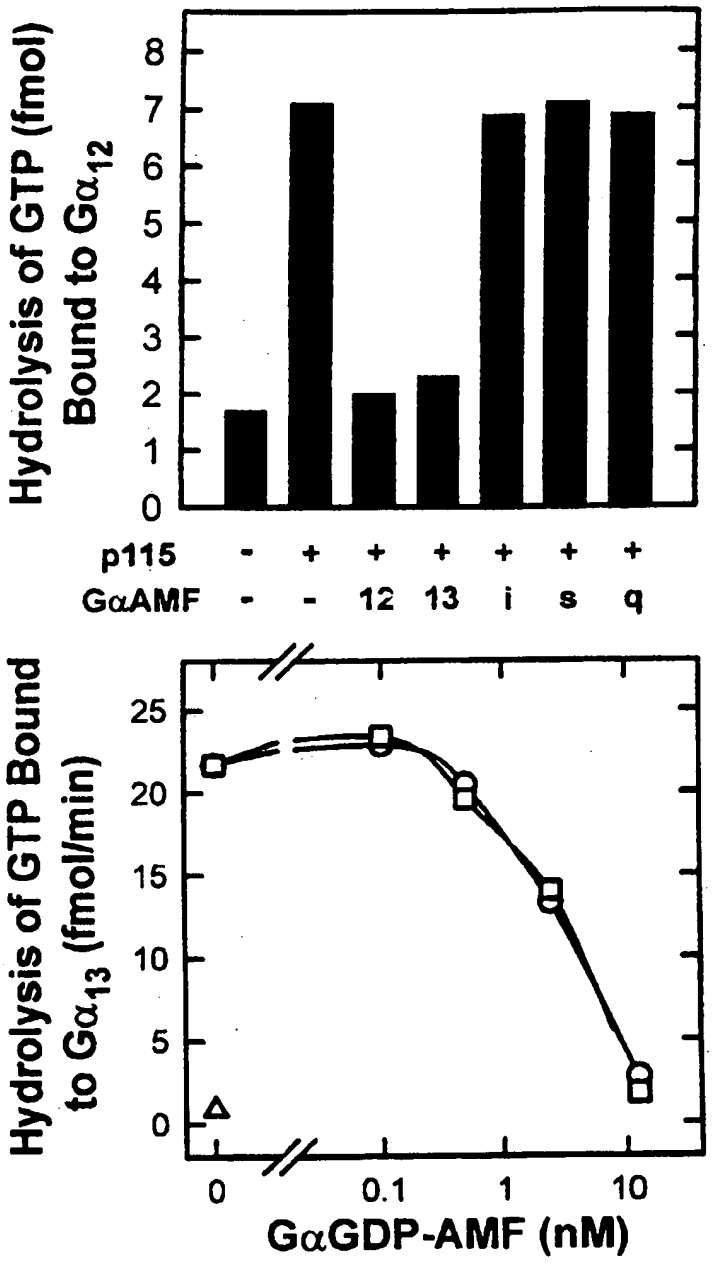

Hydrolysis of GTP (fmol) Bound to Gα12
8
7
6
5
4
3
2
1
0
p115 - + + + + + +
GαAMF - - 12 13 i s q
Hydrolysis of GTP Bound to Gα13 (fmol/min)
25
20
15
10
5
0
0
0.1
1
10
GαGDP-AMF (nM)

FIGURE 5

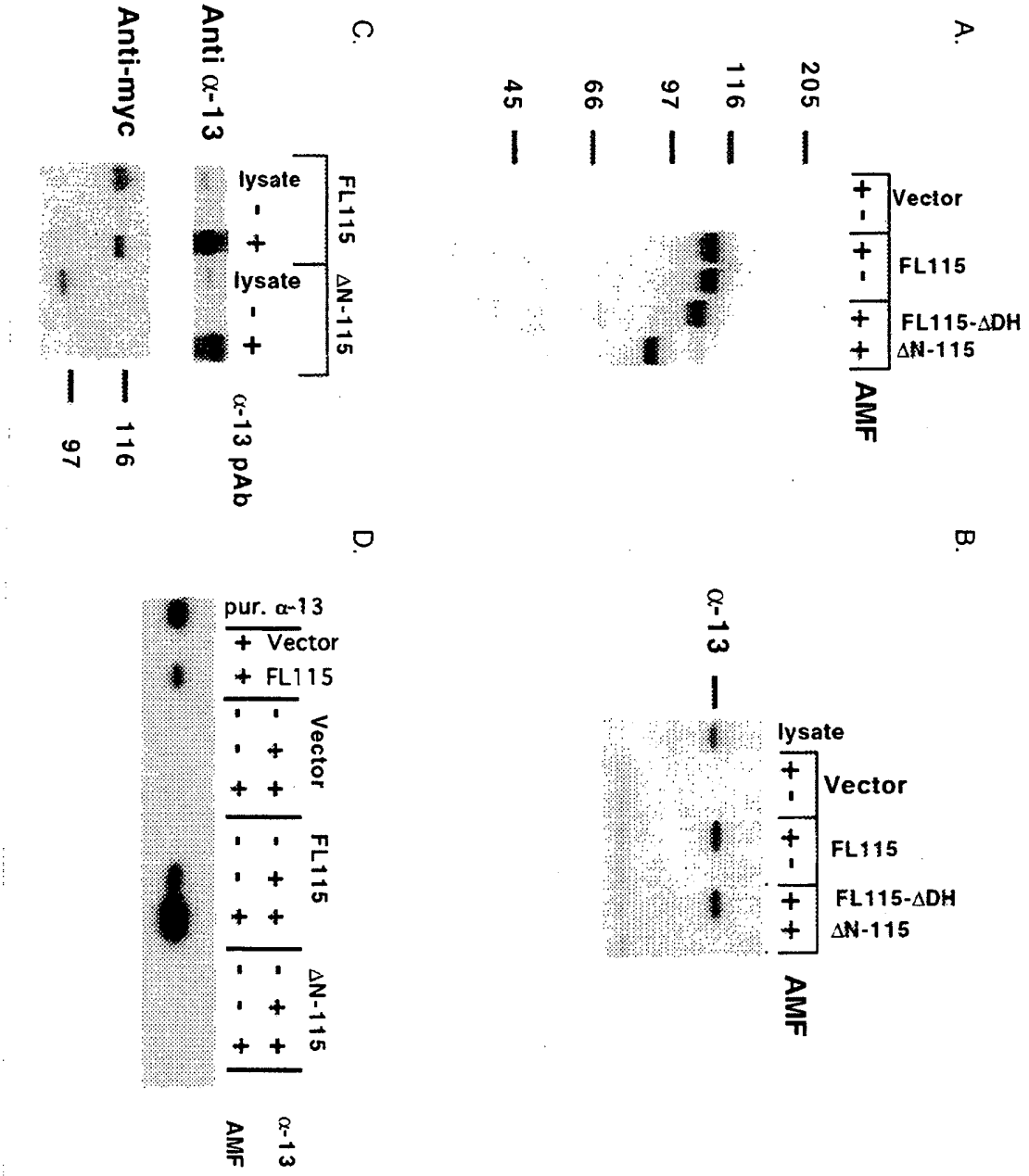

A.
205
116
97
66
45
+ - Vector
+ - FL115
+ + FL115-ΔDH
ΔN-115
AMF
B.
α-13
lysate
+ - Vector
+ - FL115
+ + FL115-ΔDH
ΔN-115
AMF
C.
Anti α-13
lysate
- + FL115 ΔN-115
lysate
- +
α-13 pAb
Anti-myc
116
97
D.
pur. α-13
+ Vector
+ FL115
- - + + Vector
- - + + FL115
- - + + ΔN-115
AMF
α-13

FIGURE 6

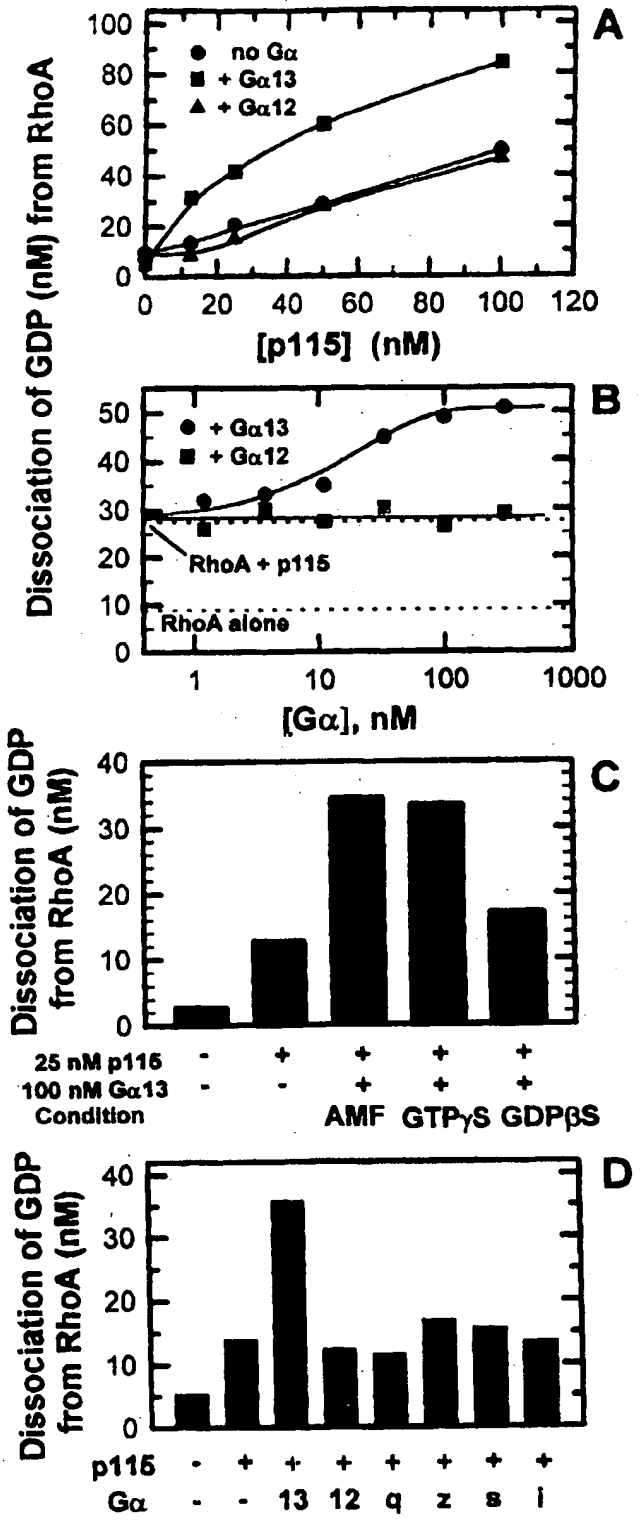

A
no Gα
+ Gα13
+ Gα12
Dissociation of GDP (nM) from RhoA
[p115] (nM)
B
+ Gα13
+ Gα12
RhoA + p115
RhoA alone
[Gα], nM
C
Dissociation of GDP from RhoA (nM)
25 nM p115
100 nM Gα13
Condition
AMF GTPγS GDPβS
D
Dissociation of GDP from RhoA (nM)
p115
Gα
13 12 q z s i

FIGURE 7

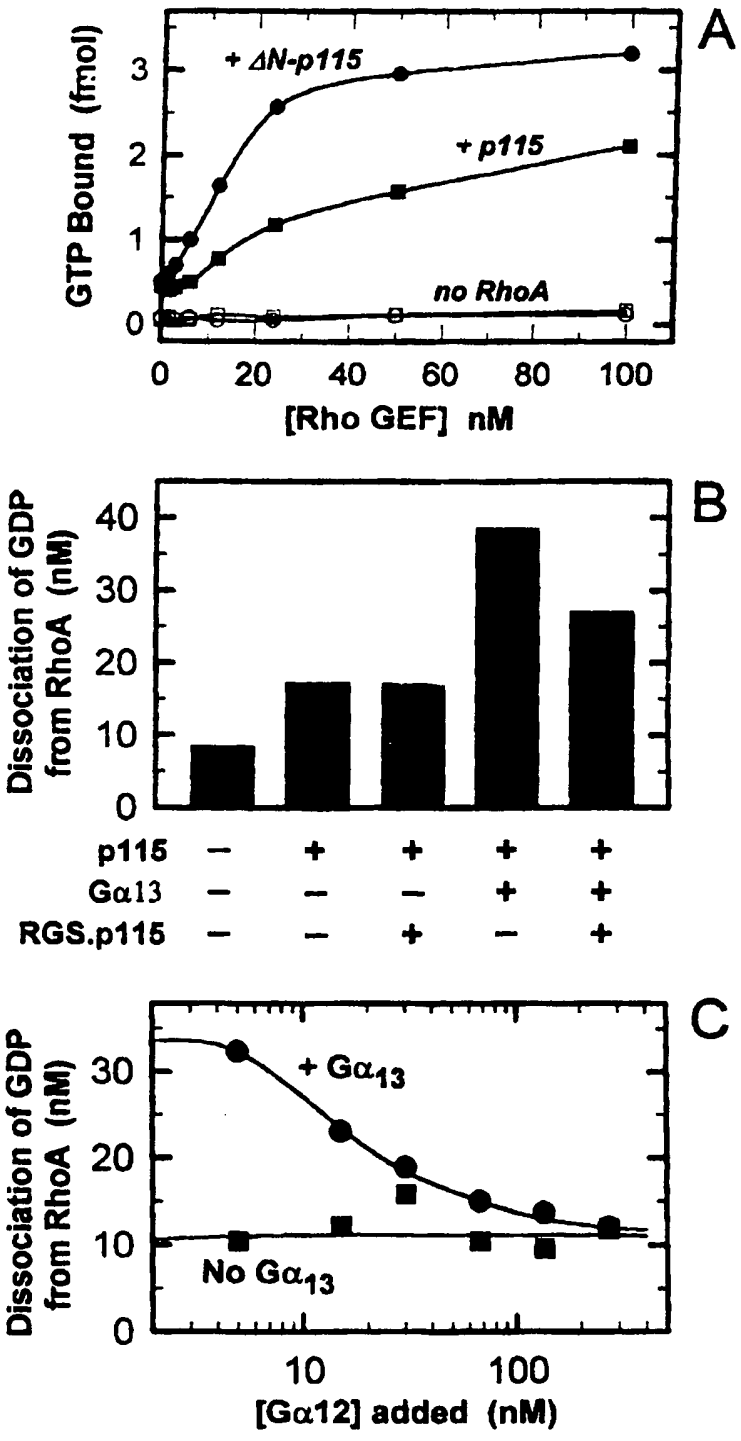

A
GTP Bound (fmol)
+ ΔN-p115
+ p115
no RhoA
[Rho GEF] nM
B
Dissociation of GDP from RhoA (nM)
p115
Gα13
RGS.p115
C
Dissociation of GDP from RhoA (nM)
+ Gα13
No Gα13
[Gα12] added (nM)

FIGURE 8

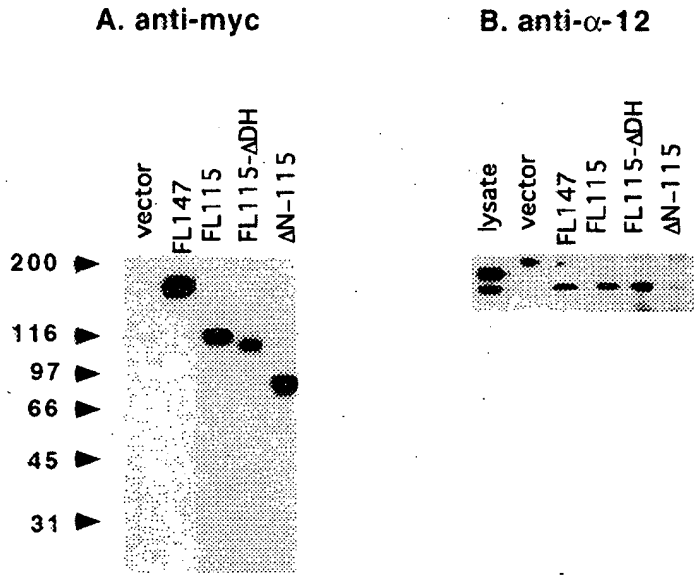

A. anti-myc
B. anti-α-12
vector
FL147
FL115
FL115-ΔDH
ΔN-115
200
116
97
66
45
31
lysate
vector
FL147
FL115
FL115-ΔDH
ΔN-115

FIGURE 9


10 20 30 40
medfargaaspgpsrpglvpvsiigaededfeneletnse 40
eqnsqfqsleqvkrrpahlmallqhvalqfepgpllcclh 80
admlgslgpkeakkafldfyhsflektavlrvpvppnvaf 120
eldrtradlisedvqrrfvqevvqsqqvavgrqledfrsk 160
rlmgmtpweqelaqleawvgrdrasyearerhvaerllmh 200
210 220 230 240
leemqhtistdeeksaavvnaiglymrhlgvrtksgdkks 240
grnffrkkvmgnrrsddppktkkglssildaarwnrgepq 280
vpdfrhlkaevdaekpgatdrkggvgmpsrdrnigapgqd 320
tpgvslhplsldspdrepgadaplelgdsspqgpmslesl 360
appestdegaetespepgdegepgrsglelepeeppgwre 400
410 420 430 440
lvppdtlhslpksqvkrqevisellvteaahvrmlrvlhd 440
lffqpmaeclffpleelqnifpsldelievhslfldrlmk 480
rrqesgylieeigdvllarfdgaegswfqkissrfcsrqs 520
faleqlkakqrkdprfcafvqeaesrprcrrlqlkdmipt 560
emqrltkyplllqsigqnteepterekvelaaeccreilh 600
610 620 630 640
hvnqavrdmedllrlkdyqrrldlshlrqssdpmlsefkn 640
lditkkklvhegpltwrvtkdkavevhvllldddllllqr 680
qderlllkshsrtltptpdgktmlrpvlritsamtrevat 720
dhkafyvlftwdqeaqiyelvaqtvserknwcalitetag 760
slkvpapasrpkprprpsstrepllsssengnggretspa 800
810 820 830 840
darterilsdllpfcrpgpegqlaatalrkvislkqllfp 840
aeedngagpprdgdgvpgggplspartqeiqenllsleet 880
mkqleeleeefcrlrpllsqlggnsvpqpgct 912

FIGURE 10

```
        10         20         30         40
gggcgccccgccggtcacttccgcgcggacaccagccttg 40
cagagcccagggagatggaagacttcgcccgaggggcggc 80
ctccccaggcccctcccggcctggcctggttcccgtcagc 120
atcatcggggctgaggatgaggattttgagaacgagctgg 160
agacaaactcagaagagcaaaacagccagttccagagcct 200
        210        220        230        240
ggagcaggtgaagcggcgcccagcccacctcatggccctc 240
ctgcagcacgtggccctgcagtttgagccaggacccctgc 280
tttgctgtctgcatgccgacatgctgggctcactgggccc 320
caaggaggccaagaaggccttcctggacttctaccacagc 360
ttcctggagaagacagcggttctccgggtgccggtccctc 400
        410        420        430        440
ccaacgtcgcctttgaacttgaccgcactagggctgacct 440
catctccgaggatgtccagcggcggttcgtgcaggaggtg 480
gtgcaaagccagcaggtagccgtgggccggcagctggagg 520
acttccgttccaagcggctcatgggcatgacgccctggga 560
gcaggagctggcccagctggaggcttgggttgggcgggac 600
        610        620        630        640
cgagccagctacgaggcccgggagcggcacgtggcggagc 640
ggctgctcatgcacctggaggagatgcaacataccatctc 680
taccgacgaagaaaagagtgctgccgtggtcaacgccatt 720
gggctgtacatgcgccaccttggggtgcggaccaagagtg 760
gagacaagaagtcggggaggaacttcttccggaaaaaggt 800
        810        820        830        840
gatggggaaccggcggtcggacgaccctcccaagaccaag 840
aaggggctgagcagcatcctggatgccgcccgctggaacc 880
ggggagagccccaggttccagattttcgacacctcaaagc 920
agaggttgatgccgagaagccaggtgctacagaccggaag 960
ggaggcgtggggatgccctctcgggaccggaatatcgggg 1000
        1010       1020       1030       1040
ctcctgggcaggacacccctggagtctctctgcaccctct 1040
gtccctggacagcccagaccgggaaccaggtgctgacgcc 1080
cccctggagctgggggactcatccccgcagggcccaatga 1120
gcctggagtccttggcgcccccagagagtaccgacgaggg 1160
ggccgaaaccgagagccccgagcctggagatgaggggggag 1200
        1210       1220       1230       1240
ccggggcggtcgggactggagcttgaaccagaagagcctc 1240
ccggctggcgggaactcgtcccccccagacaccctgcacag 1280
cctgcccaagagccaggtgaagcggcaggaggtcatcagc 1320
gagctgctggtgacagaggcggcccacgtgcgcatgctgc 1360
gggtgctgcacgacctcttcttccagcccatggcagaatg 1400
```

FIGURE 11

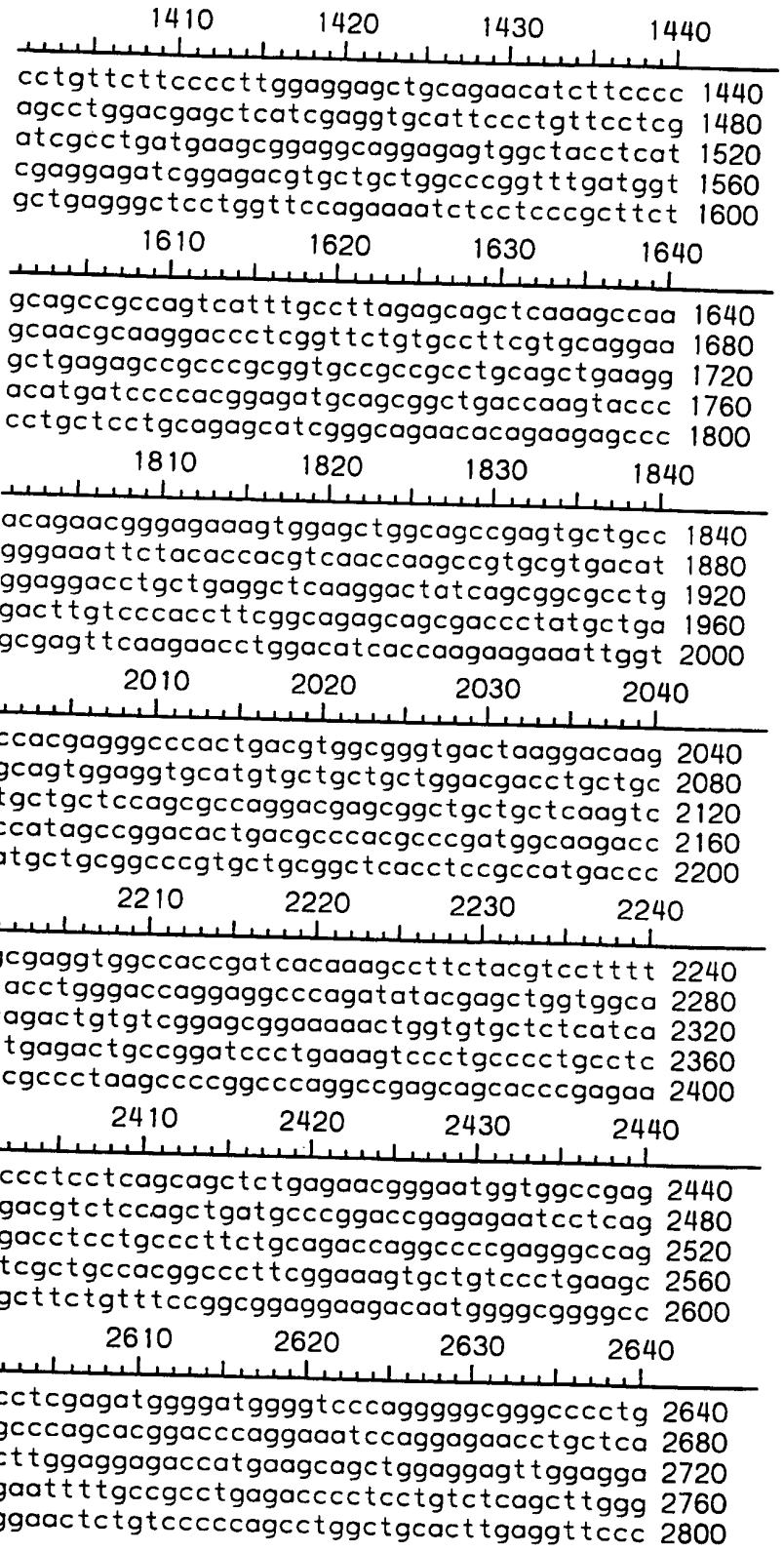


FIGURE 11 cont.

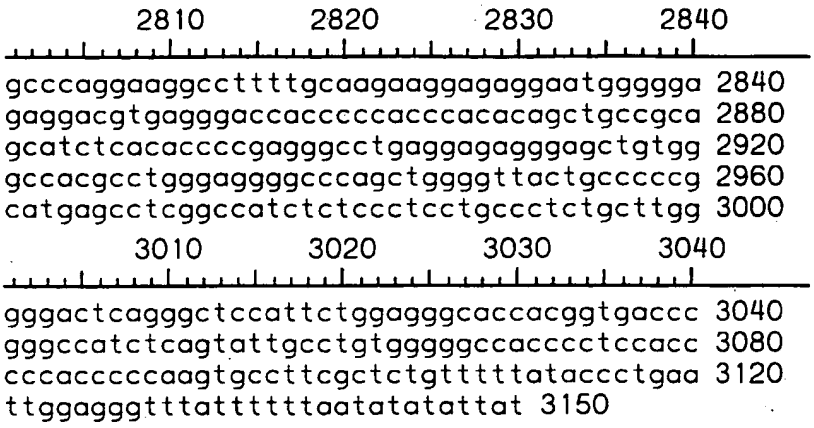


FIGURE 11 cont.


10 20 30 40
msvrlpqsidrlssls lgdsaperkspshhrqpsdaset 40
tglvqrcviiqkdqhgfgftvsgdrivlvqsvrpggaamk 80
agvkegdriikvngtmvtnsshlevvkliksgayvaltll 120
gsspssmgisglqqdpspagapritsvipsppppppplppp 160
qritgpkplqdpevqkhatqilrnmlrqeekelqdilply 200
210 220 230 240
gdtsqrpsegrlsldsqegdsgldsgterfpslseslmnr 240
nsvlsdpgldsprtspvimarvaqhhrrqgsdaavpstgd 280
qgvdqspkpliigpeedydpgyfnnesdiifqdlekliksr 320
pahlgvflryifsqadpspllfylcaevyqqaspkdsrsl 360
gkdiwnifleknaplrvkipemlqaeidsrlrnsedargv 400
410 420 430 440
lceaqeaampeiqeqihdyrtkrtlglgslygendlldld 440
gdplrerqvaekqlaalgdilskyeedrsapmdfalntym 480
shagirlrearpsntaekaqsapdkdkwlpffpktkkssn 520
skkekdaledkkrnpilkyigkpkssqstfhiplspvev 560
kpgnvrniiqhfennqqydapepgtqrlstgsfpedlles 600
610 620 630 640
dssrseirlgrseslkgreemkrsrkaenvprsrsdvdmd 640
aaaeatrlhqsasssts slstrslenptppftpkmgrrsi 680
espslgfctdtllphlleddlgqlsdlepepdaqnwqhtv 720
gkdvvagltqreidrqevinelfvteashlrtlrvldlif 760
yqrmkkenlmpreelarlfpnlpelieihnswceamkklr 800
810 820 830 840
eegpiikeisdlmlarfdgpareelqqvaaqfcsyqsial 840
eliktkqrkesrfqlfmqeaeshpqcrrlqlrdliisemq 880
rltkyplllesiikhteggtsehekicrardqcreilkyv 920
neavkqtenrhrlegyqkrldatalerasnplaaefksld 960
lttrkmihegpltwriskdktldlhvllledllvllqkqd 1000
1010 1020 1030 1040
ekllikchsktavgssdskqtfspvlklnavlirsvatdk 1040
raffiictsklgppqiyelvaltssdkntwmelleeavrn 1080
atrhpgaapmpvhppppgprepaqqgptpsrveldddsdvf 1120
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isvtshpwdpgspgqappggegdntqlaglegerpeqedm 1280
glcslehlpprtrnsgiwespeldrnlaedassteaaggy 1320
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fyvsmpsgppdsstdhseapmsppqpdslpagqtepqpql 1400

FIGURE 12

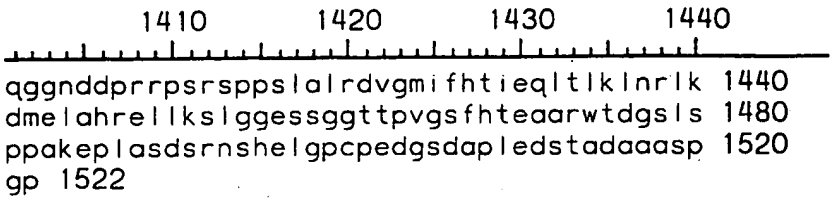

FIGURE 12 cont.

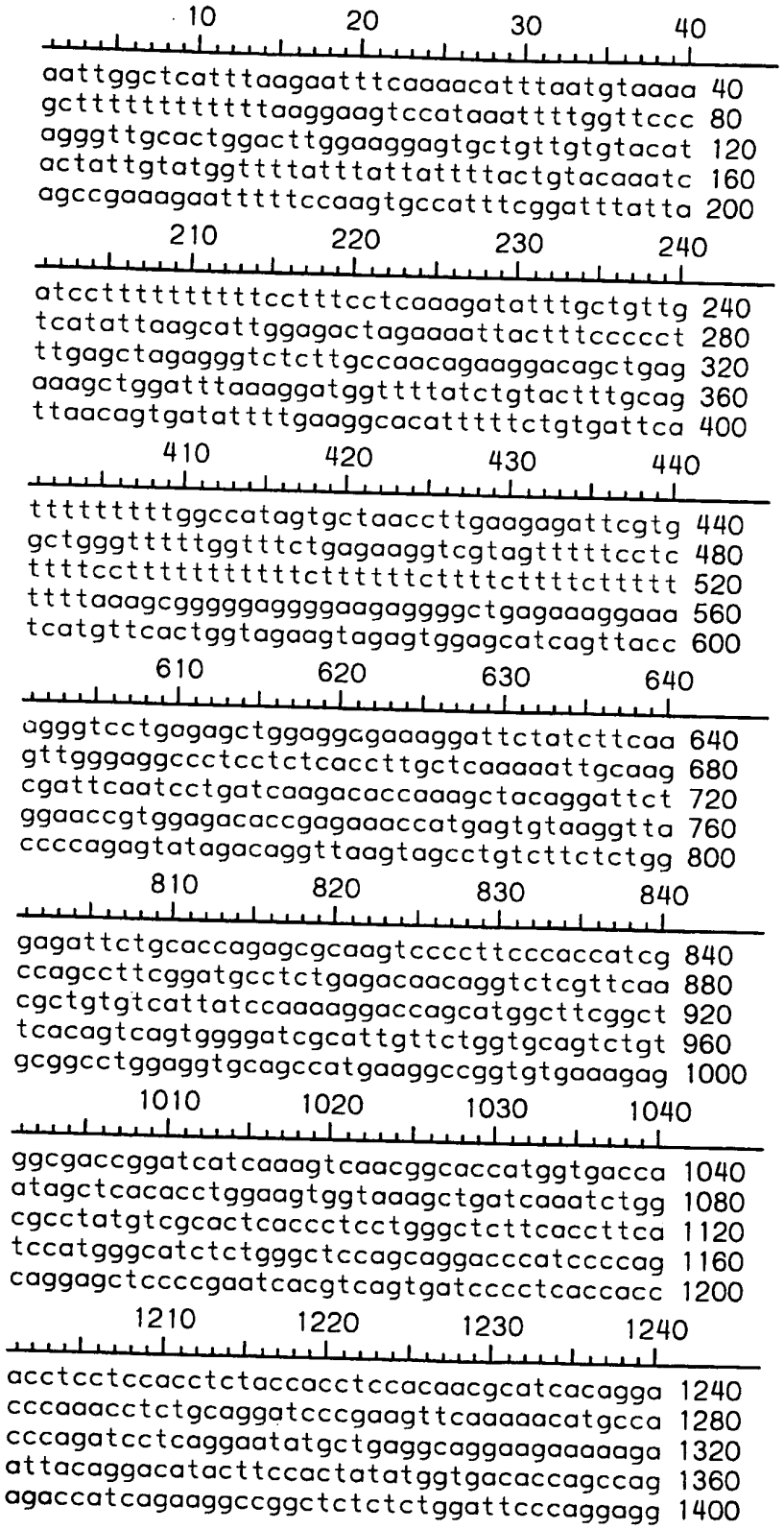
10 20 30 40
aattggctcatttaagaatttcaaaacatttaatgtaaaa 40
gctttttttttttaaggaagtccataaattttggttccc 80
agggttgcactggacttggaaggagtgctgttgtgtacat 120
actattgtatggttttatttattattttactgtacaaatc 160
agccgaaagaatttttccaagtgccatttcggatttatta 200
210 220 230 240
atccttttttttttcctttcctcaaagatatttgctgttg 240
tcatattaagcattggagactagaaaattactttcccct 280
ttgagctagagggtctcttgccaacagaaggacagctgag 320
aaagctggatttaaaggatggttttatctgtactttgcag 360
ttaacagtgatattttgaaggcacatttttctgtgattca 400
410 420 430 440
ttttttttggccatagtgctaaccttgaagagattcgtg 440
gctgggtttttggtttctgagaaggtcgtagtttttcctc 480
ttttcctttttttttttctttttttctttttctttttttt 520
ttttaaagcgggggaggggaagaggggctgagaaaggaaa 560
tcatgttcactggtagaagtagagtggagcatcagttacc 600
610 620 630 640
agggtcctgagagctggaggcgaaaggattctatcttcaa 640
gttgggaggccctcctctcaccttgctcaaaaattgcaag 680
cgattcaatcctgatcaagacaccaaagctacaggattct 720
ggaaccgtggagacaccgagaaaccatgagtgtaaggtta 760
ccccagagtatagacaggttaagtagcctgtcttctctgg 800
810 820 830 840
gagattctgcaccagagcgcaagtccccttcccaccatcg 840
ccagccttcggatgcctctgagacaacaggtctcgttcaa 880
cgctgtgtcattatccaaaaggaccagcatggcttcggct 920
tcacagtcagtggggatcgcattgttctggtgcagtctgt 960
gcggcctggaggtgcagccatgaaggccggtgtgaaagag 1000
1010 1020 1030 1040
ggcgaccggatcatcaaagtcaacggcaccatggtgacca 1040
atagctcacacctggaagtggtaaagctgatcaaatctgg 1080
cgcctatgtcgcactcaccctcctgggctcttcaccttca 1120
tccatgggcatctctgggctccagcaggacccatccccag 1160
caggagctccccgaatcacgtcagtgatcccctcaccacc 1200
1210 1220 1230 1240
acctcctccacctctaccacctccacaacgcatcacagga 1240
cccaaacctctgcaggatcccgaagttcaaaaacatgcca 1280
cccagatcctcaggaatatgctgaggcaggaagaaaaaga 1320
attacaggacatacttccactatatggtgacaccagccag 1360
agaccatcagaaggccggctctctctggattcccaggagg 1400

FIGURE 13

```
        1410        1420        1430        1440
gggacagtggcttggactctgggacagaacgctttccttc 1440
cctcagtgagtcattgatgaatcggaactcggtactgtca 1480
gaccctgggctagacagtcctcgaacctcccctgtgatca 1520
tggccagggtggcccagcaccacaggcggcagggctcgga 1560
tgcagcagtcccctcaaccggtgaccagggtgtagatcaa 1600
        1610        1620        1630        1640
agcccaaagcctttaattattggcccagaggaagactatg 1640
acccgggttatttcaacaacgagagcgacatcatattcca 1680
ggatctggagaaactgaagtctcggccagctcacctgggg 1720
gtttttctacgttacatcttctctcaggcggaccccagtc 1760
cactgctttttacctgtgtgcagaagtttatcagcaggc 1800
        1810        1820        1830        1840
aagccccaaggattcccgaagcttggggaaagacatctgg 1840
aatattttcctggagaaaaatgcgcctctgagagtgaaga 1880
tccctgagatgctacaggctgaaattgactcgcgcctgcg 1920
gaacagcgaagatgcccgtggtgttctctgtgaagctcaa 1960
gaggcagccatgcctgagatccaagagcagatccacgact 2000
        2010        2020        2030        2040
acagaacgaagcgcacactggggctgggcagcctgtatgg 2040
tgaaaatgacctgctggacctggatggggaccctctccga 2080
gagcgccaagtggctgagaagcagctggctgcccttggag 2120
atattttgtccaagtatgaggaagacaggagcgcccccat 2160
ggacttcgccctcaatacctacatgagccatgctgggatc 2200
        2210        2220        2230        2240
cgtcttcgagaggcacgaccttccaacacagctgaaaagg 2240
cccagtctgctcctgacaaggacaagtggctaccgttctt 2280
ccctaagaccaagaagagcagcaattccaagaaagaaaag 2320
gatgccttggaggacaagaagcgaaaccctatcctcaaat 2360
acattgggaagcccaaaagctcttctcaaagcacatttca 2400
        2410        2420        2430        2440
tattcccttgtcccctgtggaagtcaaaccaggcaatgtg 2440
aggaacatcattcagcactttgagaacaaccagcagtatg 2480
atgccccagaacctgggacacaacgactctcgaccggaag 2520
ctttcctgaggacctgctggagagtgacagttcacgctca 2560
gagattcgcctgggccgctctgaaagcctcaagggccggg 2600
        2610        2620        2630        2640
aagagatgaaacggtctcgaaaggcagagaacgtgccccg 2640
ctctcgcagtgatgttgacatggatgctgctgcggaggct 2680
actcgcctgcaccagtcagcctcgtcctctacctccagcc 2720
tctccaccaggtctcttgagaacccaaccctccattcac 2760
tcccaaaatgggccgcaggagcattgagtcccccagtttg 2800
```

FIGURE 13 cont.

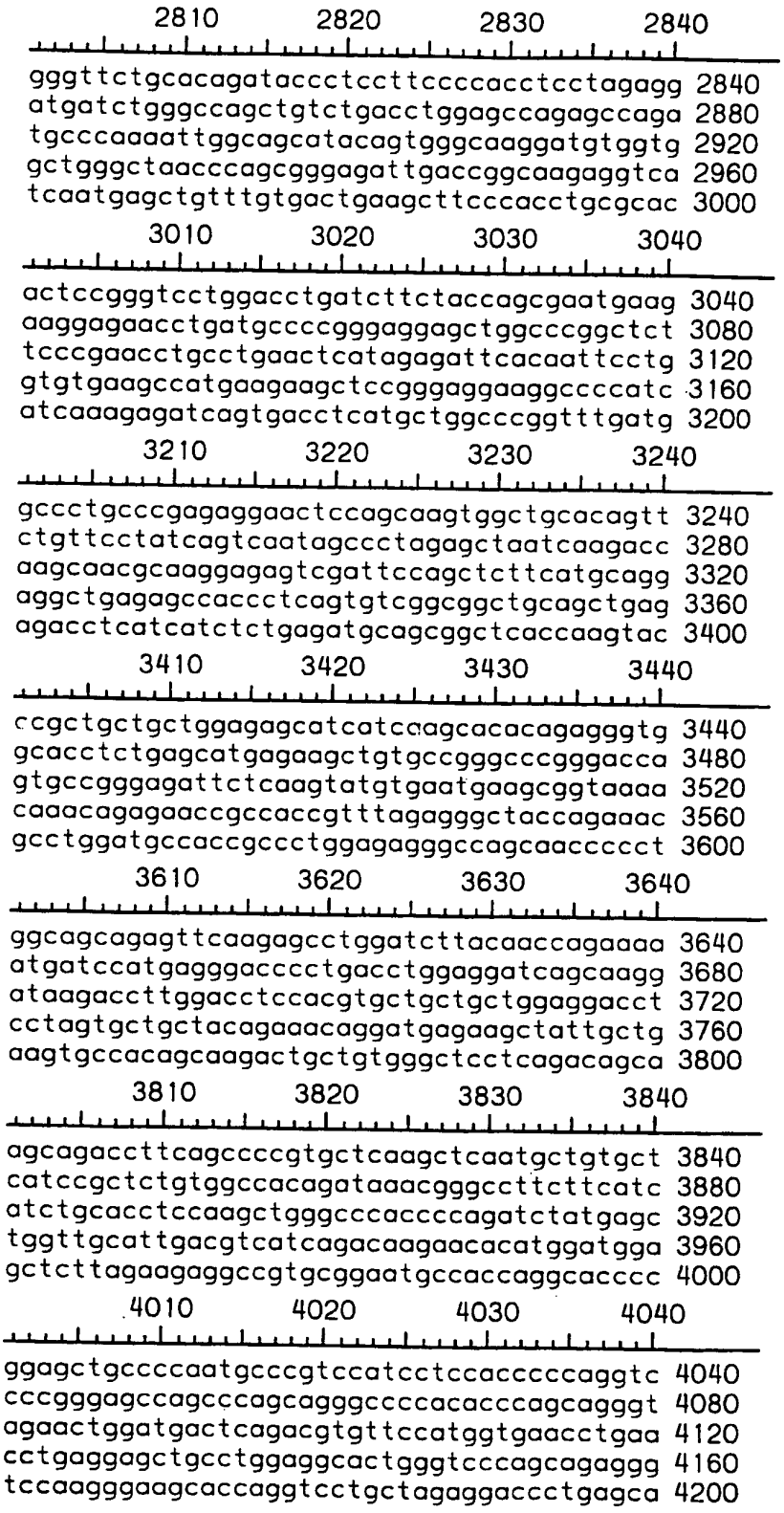

FIGURE 13 cont.

```
        4210        4220        4230        4240
ggagggcagtgcagaggaagaggaactgggtgtcctgcct 4240
tgcccttccacatccctggatggagagaacaggggcatca 4280
ggacaaggaaccccatccacttggccttcccaggccctct 4320
gttcatggaagggctcgctgactccgctctggaagatgtg 4360
gagaacctgcgacatctgatcctgtggagcctgctgccag 4400
        4410        4420        4430        4440
gtcacaccatggaaactcaggctgcccaggagcccgagga 4440
cgacctgacacccacaccttctgtcatcagcgtcacctct 4480
caccccctgggacccaggctccccagggcaagcacccctg 4520
ggggtgaaggggacaacacccagcttgcagggctggaggg 4560
ggaacggccagagcaggaagacatgggtctctgttctctg 4600
        4610        4620        4630        4640
gaacacctacccccaaggaccagaaattctgggatatggg 4640
agtctccagaactggacaggaatctggctgaagatgcttc 4680
aagcacagaggcagcaggaggttacaaagttgtgagaaaa 4720
gctgaggtggcaggcagcaaggttgtccctgcactaccag 4760
agagtggccagtcagagcctgggccacctgaagtggaagg 4800
        4810        4820        4830        4840
cggaacaaaggctacggggaactgcttttatgtcagcatg 4840
ccatcaggacccccggactcaagcaccgaccactcagagg 4880
cacccatgagccccccctcagcctgacagcctccctgcagg 4920
gcagacagagcctcagcctcagctgcagggaggcaacgat 4960
gatccaagacgccccagccgctctcctccaagcctggccc 5000
        5010        5020        5030        5040
tcagggacgtgggcatgatcttccataccattgagcagct 5040
cactctcaagctcaacaggctcaaggatatggagctggcc 5080
cacagagagctgctcaagtcccttgggggagagtcatctg 5120
gtggcaccacgcctgtgggcagtttccacacagaagcagc 5160
tagatggacagatggctccctctcacctcccgctaaggag 5200
        5210        5220        5230        5240
cccctagcttctgactccaggaacagccatgaactggggc 5240
cctgccctgaggatggctctgacgcccccctggaagacag 5280
cacagcagacgcagccgcgtcaccaggaccataaccgtac 5320
aaaccaccaaatcctctgcgtccccactcctccttcaggg 5360
actggcctgagaccggggcacagggtagggggatcccaa 5400
        5410        5420        5430        5440
cactcctccctgtggaggaggcagttagggaaactaggat 5440
ccagccaaggcccgggggagacccgcatgttgcttggtc 5480
tgctcaagtcggagtcaggtttcagtgtcttttccctccc 5520
ttagcccaaccctccaaggcctcatgtctcctaagcatgc 5560
tgactgcatccgaaaggcccccactcaccatggtctgccc 5600
```

FIGURE 13 cont.

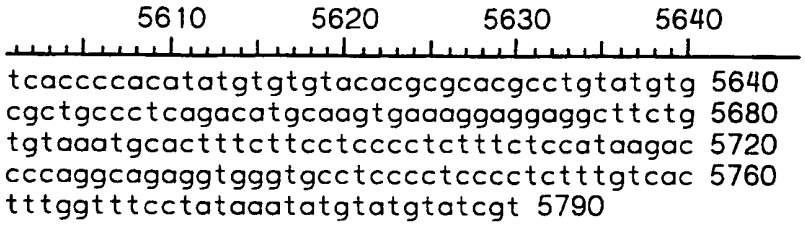

FIGURE 13 cont.

```
          10        20        30        40
          |         |         |         |
mgevaggaapgpprsglvsiiigaededfeneleansedq 40
nsqfqsleqvkrrpahlmallqhvalqfepgpllcclhad 80
mlsslgpkeakkafldfyhsflektavlrvpvppsvafel 120
drtrpdlisedvqrrfiqevvqsqqaavsrqledfrskrl 160
mgmtpweqelsllepwigkdrgnyearerhvaerllshle 200
        210       220       230       240
        |         |         |         |
etqhtistdeeksaavvtaislymrhlgvrtksgdkksgr 240
nffrkkvmgnrrsdeppktkkglssildparwnrgepsap 280
dcrhlkveadekpgpadrkgglgmssrdrtvgtpgqdnpg 320
vslhplstdsvdsrepgvdtpqepgdtppqgptsleplap 360
pestedngetespepgddgepgrsglelepeeppgwrelv 400
        410       420       430       440
        |         |         |         |
ppdtllslpksqvkrqevisellvteaahvrmlrvlhdlf 440
yqpmadggffpldelqnifpsldelievhslfldrlmkrr 480
qesgylieeigdvllarfdgaegswfqkissrfcsrqsfa 520
leqlkakqrkeprfcafvqeaesrprcrrlqlkdmiptem 560
qrltkypllqsigqnteestergkvelaaeccreilhhv 600
        610       620       630       640
        |         |         |         |
nqavrdmedllrlkdyqrrldlthlrqssdpmlsefknld 640
itkkklvhegpltwrvtkdkaievhvlllddlllllqrqd 680
erlllkshsrtltptpdgktmlrpvlrltsamtrevatdh 720
kafyviftwdqeaqiyelvaqtsserknwcnlitetagsl 760
kvpapasrlkprpspssirepllsssengtggaemapada 800
        810       820       830       840
        |         |         |         |
rterllndllpfcrpgpegqlaatalqkvslslkqillste 840
edsgagpprdgdgvpggrapgpvhtqeieenllslevair 880
qleeleeefcrlrpllsqlggtlspnlaapersaqtgls 919
```

FIGURE 14


10 20 30 40
cgggacaccgggcccgggatccccgagcccgacctcgggc 40
gcctcgccggtcacctccccgcgcggacaccagcccggcg 80
gagtcctggagatgggagaagtcgccggaggggcggcccc 120
agggcctccccggtctggcctggtgtccatcatcatcggg 160
gcggaggatgaggattttgagaacgagctggaggcgaact 200
210 220 230 240
cagaagatcaaaacagccagttccagagcctagagcaagt 240
gaagcgccgccctgcccacctcatggccctcctgcagcat 280
gtggccctgcagttcgagccaggaccactgctctgctgcc 320
tgcatgcagacatgctgagctctctgggccccaaagaagc 360
caagaaggccttccttgacttctatcacagtttcctggag 400
410 420 430 440
aagactgcggttctacgggtgccggtccctcccagtgtcg 440
cttttgaacttgatcgtactcgacctgatctgatctctga 480
ggatgtccagaggcggttcatacaagaggtggtgcagagc 520
cagcaggcagccgtgagccgtcagctagaggacttccgct 560
ccaagcggctcatgggcatgacgccctgggagcaggaact 600
610 620 630 640
gagcctgctggagccctggattgggaaagacccgaggcaac 640
tatgaggcccgggagcggcatgttgcggagcggctgctgt 680
cccacctggaggagacccagcataccatctctacagatga 720
agagaaaagtgctgctgtggtcactgccatcagcctgtat 760
atgcgccaccttggagtccggaccaagagtggggacaaga 800
810 820 830 840
agtcgggaaggaacttcttccggaaaaaggtgatggggaa 840
tcggaggtcagacgaacccccaaagacaaagaaagggctg 880
agcagtatcctagatcctgcacgttggaaccggggagagc 920
catccgctccagattgtcgacatctaaaggtcgaggctga 960
tgagaagccaggccctgcagaccggaagggaggcctgggt 1000
1010 1020 1030 1040
atgtcttctcgggacaggactgttgggactcctggacagg 1040
acaacccaggagtctccctgcaccctctgtctacagacag 1080
cgtcgactcccgggaaccaggcgtggataccccgcaggag 1120
ccaggggatacacccccacagggccctaccagcctggagc 1160
ccctggcgcccccagagagcacagaggacaatggcgagac 1200
1210 1220 1230 1240
tgagagccctgagcccggagatgatggggagccaggacgg 1240
tcaggcctggaactggaaccagaagaacctcctgggtgga 1280
gggaactcgtgccccccagacaccctgctcagtctgcccaa 1320
gagccaagtgaagcggcaagaggtcatcagcgagctgctc 1360
gtgactgaggcagctcacgtgcgcatgctacgggtactgc 1400

FIGURE 15

```
      1410       1420       1430       1440
atgacctcttctaccagcccatggcggatggaggcttctt 1440
ccctctggacgagctgcagaacatcttcccgagcctggat 1480
gagctcatcgaggtgcactccctgttcctcgatcgcttga 1520
tgaagcggagacaagagagtggctacctcattgaggagat 1560
cggcgatgtgctactggcccggttcgatggtgctgagggc 1600
      1610       1620       1630       1640
tcatggttccagaagatctcctcccgcttctgcagccgcc 1640
agtcgttcgctctagagcagctcaaagccaagcagcgcaa 1680
ggagcctcggttctgtgcctttgtgcaggaagctgagagc 1720
cgcccgagatgccggcgcctacagttaaaggacatgatcc 1760
ccactgagatgcagcgactgaccaagtacccactgctgct 1800
      1810       1820       1830       1840
acagagcatcgggcagaacacagaggagtctacagaacga 1840
gggaaagtggagcttgcagctgagtgctgccgggagattc 1880
tgcaccatgtcaatcaagccgtccgtgacatggaggacct 1920
gctgcggctcaaggattaccagcggcgcctggacttgact 1960
cacctacggcagagcagtgaccctatgctgagcgagttca 2000
      2010       2020       2030       2040
agaacctggacatcactaagaagaagttggtccatgaagg 2040
ccccctcacgtggcgagtgaccaaagacaaagctatagaa 2080
gtgcacgtgctcttgctggacgacctgctgctgctgctcc 2120
agcgccaggacgagaggctgctgctcaagtcccacagccg 2160
gacgctgacacctacccccgatggcaagaccatgctgcgg 2200
      2210       2220       2230       2240
ccggtgctccggctcacctctgccatgacccgagaggtgg 2240
ccactgatcacaaagctttctacgtcatttttacctggga 2280
ccaggaggcccagatatatgagctggtggcacagacatct 2320
tcggaacgcaaaaactggtgtaacctcatcactgagactg 2360
ctggatccctgaaggtccctgcccctgcctcccgcctcaa 2400
      2410       2420       2430       2440
accccggcccagcccaagcagcatccgagaacccctgctc 2440
agcagctctgagaatggcactggaggcgcagagatggctc 2480
cagctgatgccaggacagagcggctcctcaatgacctcct 2520
gcccttctgcagaccaggcccagagggccagcttgctgcc 2560
acagcccttcagaaagtactgtccctgaagcagatcctgc 2600
      2610       2620       2630       2640
taagcactgaggaagacagtggagcggggcctccccgcga 2640
tggggatggggtgcctggtggtagggcccccggcccagtg 2680
cacacccaggagattgaggaaaacttgcttagcttagagg 2720
tggccatcagacaactggaggagttggaagaggaattttg 2760
tcgcctaagacccctcctgtcccagcttgggggactctg 2800
```

FIGURE 15 cont.

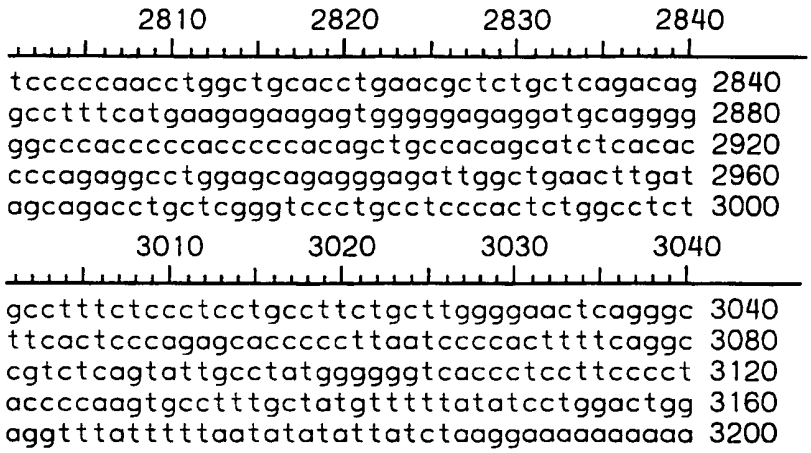

FIGURE 15 cont.

```
          10        20        30        40
mddpsikkrlldlytdeheydevqeipeessiqppetsts 40
htstngsshsgpgtatgpgatsagpsagapqspvivvdsv 80
pelpapkqksvknskskqkqkqlankskiprspslassls 120
slasslsghrdrdkdrdkdrenqnavppqtpplppsykqn 160
qmngdstaaagggvsapatpttannnnashnngsimgggv 200
          210       220       230       240
qlnqsdnsnpvlqapgerssslnltplsrdlsgghtqestt 240
pattpstpslalpknfqyltltvrkdsngygmkvsgdnpv 280
fvesvkpggaaeiaglvagdmilrvnghevrlekhptvvg 320
likasttvelavkrsqkltrpssvsvvtpstpilsgrdrt 360
asitgpqpvdsikrremetykiqtlqkmleqeklnlerlk 400
          410       420       430       440
sdqnnpsyklseanirklreqlhqvgaedaptvklqaaag 440
nkntallтpnqiqhlsasathsnqqfhhlhhhhnlhnnny 480
ppqqqpastspaflsllprslsslslgtrknktekdltts 520
spfglttdflqqqrmshqaesmsqsmhqhtstptsqqffh 560
phqqqhrfketgptskgknkflisrslieedvppplpqrn 600
          610       620       630       640
pprqlnldlkngnaspggshlvapvsdldratspqlnrsq 640
qqqlprstdnspsnakskrskiktkalsdpkmstqmllqm 680
esasaagaaggsievdggpppplpprlpgmmtedmsrgscq 720
nlaqpnsvgtafnyplvstttavqndnlniafplsqrpni 760
vqqlqqyqqqqqhqmsggqatgalgqtpnlgknkhrrvgs 800
          810       820       830       840
spdnmhprhpdritkttsgsweivekdgessppgtpplpy 840
lssshmtvledpnennrgaaaagpgvfieshqftpmagas 880
spipislhsshmhaaqsndtqkeiismedensdldepfid 920
engpfnnltrlleaenvtflaiflnyvisnsdpapllfyl 960
itelykegtskdmrkwayeihstflvpraplswyrqdesl 1000
          1010      1020      1030      1040
arevdnvlqleydkveilrtvflrsrkrakdliseqlref 1040
qqkrtaglgtiygptddklaeaktdklreqiidkylmpnl 1080
haliedengsppedvrkvalcsalstviyrifntrpppss 1120
ivervhhfvsrdksfksrimgknrkmnvrghplvlrqyye 1160
vthcnhcqtiiwgvspqgyhctdcklnihrqcskvvdesc 1200
          1210      1220      1230      1240
pgplpqakrlahndkiskfmgkirprtsdvignekrsrqd 1240
eeldveltpdrgqasivrqpsdrrpdanisirsngntscn 1280
tsglnttdlqssfhgscandsinpgggagcnmdlstsvas 1320
ttpstsgsvaaglsafaelnaldtvdkearrerysqhpeh 1360
ksapvsvnrsesykerlsnkrnrnsrrktsdpslssrpnd 1400
```

FIGURE 16

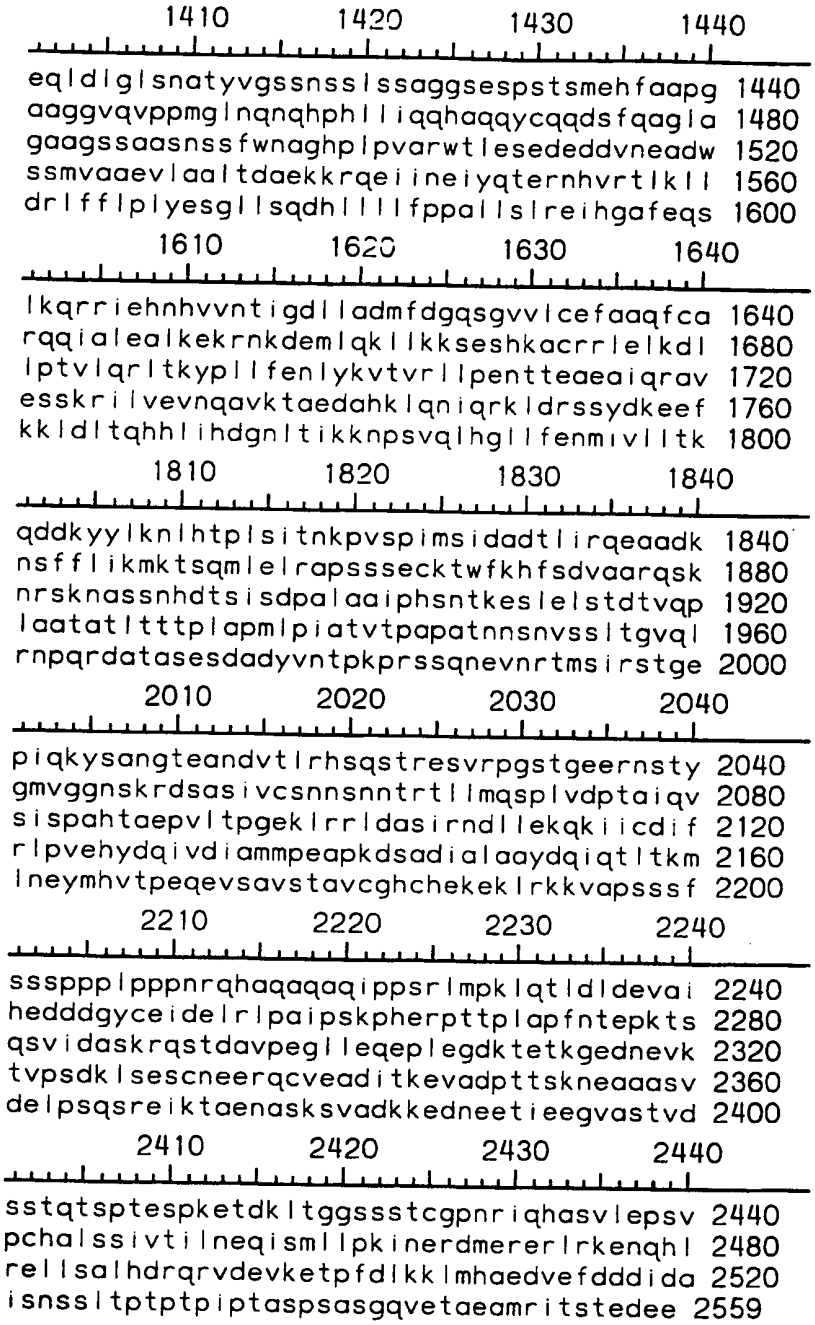

FIGURE 16 cont.

```
        10         20         30         40
aagaacgtcgtgtgcgtgttgatggcgcgataagtgaaaa 40
aaataaaaacatttttcgtgtctttatggaatacaatacg 80
agtgcgttttaagtttaagtgaaaagtagcagtgcaaaat 120
atatatacacatatcaaaacccattggaatagtgcaacca 160
aaaaaacataaaacagaactaaagcaacgaaaatcgaggc 200
       210        220        230        240
aattaaatcacttctggattcatttcggcccgtttttact 240
agctaatttaaaaatagctaccgaaagcattccgcaacca 280
cttctggtgactggccccacaccaatgcgaacaatactcc 320
acgggatcctccgacttcgagtggcttacgcatgcctttg 360
aatttgcatcttcgcacccgcacagcagccagcaaaagcc 400
       410        420        430        440
atcgcaggaaaccctatggatgacccatcaatcaaaaaac 440
ggttactagatttatatactgacgaacatgaatacgatga 480
ggttcaagagataccagaagagtcaagcattcagcccccg 520
gagacatcaacgagccataccagcacaaacggatccagcc 560
actcaggccctggaactgcaactggaccaggagcgacatc 600
       610        620        630        640
tgcaggcccgtcagcgggtgcgccacagtcaccagtaatt 640
gttgtggactcggttcccgagctgccagcgcccaagcaga 680
aatctgttaagaactcgaagagcaaacagaagcaaaagca 720
gttggcgaacaaatcgaagattccacgatctccttcgttg 760
gcgagcagcctgagtagtctggccagcagccttagtggtc 800
       810        820        830        840
atagggatcgggacaaagatcgggacaaggatcgggagaa 840
ccagaacgccgtgccgccgcagacgccgccgttgccaccg 880
agctacaagcagaaccaaatgaatggtgactctacggctg 920
cagctggtggtggtgtttctgccccagccacgcccaccac 960
cgccaataataacaatgcgagccacaacaacggcagcata 1000
      1010       1020       1030       1040
atgggcggaggcgtgcaattgaatcaatcggacaactcca 1040
accccgttctccaggcgccgggggagcgcagtagcctcaa 1080
tctgactcccctctcccgggatctgagtggtggccacacc 1120
caggagtccacgacgccagcgaccacgccgagtacgccaa 1160
gcctagcattaccaaaaaattttcagtatttaaccctgac 1200
      1210       1220       1230       1240
tgtgcgaaaagatagcaacggatacgggatgaaggtttcc 1240
ggagataatcctgtgtttgtggagagcgttaaacccggag 1280
gcgcagcggagattgcaggcctggttgctggcgatatgat 1320
actaagggtaaacggccatgaagtgcgactagagaagcat 1360
ccaactgtagtgggtcttataaaagcctcgacaactgttg 1400
```

FIGURE 17

1410 1420 1430 1440

agctggcggtgaagcgaagtcagaagctaacgcgaccctc 1440
ttcggtatcagtggtaacgccctcgacacccattctctct 1480
ggacgagatcgtaccgcttcaataactgggccacagccgg 1520
tggacagtattaaacgaagggagatggagacttacaaaat 1560
tcagacgttgcagaaaatgctggagcaggagaaactaaat 1600

1610 1620 1630 1640

ctggagcgattaaaaagcgatcaaaataatccgagctaca 1640
agctatctgaggcgaatatccgtaagctgcgcgagcaact 1680
tcatcaagtgggagctgaggatgcaccgactgttaaactt 1720
caggcggccgctggcaataagaacacagcattactaacgc 1760
ccaaccaaatccaacacttgtccgcgtccgccactcacag 1800

1810 1820 1830 1840

caatcagcaattccatcatcttcaccaccaccacaatctc 1840
cacaacaacaattatccgccacagcaacagccagctagca 1880
cctcacccgcattcctgtccctcctgccgcgttcccttтc 1920
gtccttgtcgctgggcacgcgcaaaaacaagaccgaaaag 1960
gacctaacgacttcatctccctttggcctaacgacggatt 2000

2010 2020 2030 2040

tcctgcagcagcaacggatgagccaccaagcggagtcgat 2040
gtctcaatcgatgcatcagcacaccagcactccgacctcg 2080
cagcagttcttccatcgcatcagcaacaacatcgcttta 2120
aggaaactggaccgacgtcgaaaggcaagaacaagttcct 2160
aatttcgaggagtttgatcgaggaggatgtgccgccacca 2200

2210 2220 2230 2240

ctgccgcagaggaatccgcccaggcagttaaatttggacc 2240
tgaagaacggaaacgcgtcgccgggtgggtcacatctagt 2280
ggctccagtttccgatctggatcgcgccacaagtccccaa 2320
ttaaatagatcccaacaacagcaactgcctaggagcactg 2360
acaacagcccgagcaatgccaagtccaagcgttccaagat 2400

2410 2420 2430 2440

caaaacgaaggccctatcggatcctaagatgtccactcag 2440
atgctcctgcaaatggaatcggcgagtgcagctggagcag 2480
ccggaggttccatcgaggtagatgggggtccaccaccgct 2520
accgcctcgcttgcctggcatgatgacggaggatatgagc 2560
cgcggcagctgtcagaatctcgctcaacccaattccgtgg 2600

2610 2620 2630 2640

gcactgccttcaactatcctctggtgtccacaacaacagc 2640
ggtgcagaacgataacctgaacattgcctttcctttgtcc 2680
caacgacccaacattgtccagcagctgcagcaatatcaac 2720
agcagcagcagcatcaaatgagcggtggccaggccacagg 2760
cgctctgggacagactccgaatttgggaaaaaacaagcat 2800

FIGURE 17 cont.

2810 2820 2830 2840

cgacgcgttggttcttcaccggacaatatgcatccacgtc 2840
atccagatcggataacgaaaacgacttcgggctcgtggga 2880
gattgttgaaaaggatggcgaatcctccccgcccggaaca 2920
ccgccgcttccatatctatccagctcccacatgaccgtgc 2960
tggaagatccgaatgagaacaatcgtggagcagcagcagc 3000

3010 3020 3030 3040

cggacctggagtctttatcgagtcgcatcagtttacgccg 3040
atggcgggagcctcttctccgatcccgatatccttacatt 3080
ccagccacatgcatgcggcccagtcgaacgatacgcagaa 3120
ggagatcatctcgatggaggacgaaaactcggacttggat 3160
gagcccttcattgacgagaacggacccttaacaatctaa 3200

3210 3220 3230 3240

ctcgtttgttagaggccgagaacgtcactttcctagccat 3240
cttcctaaactacgtgatctcaaactcggatcccgcgcca 3280
cttctgttttacctgattactgagttgtacaaggagggca 3320
cctccaaggacatgcggaaatgggcctacgaaatccactc 3360
cacattcctcgtgccgcgggctccattgtcatggtatcgc 3400

3410 3420 3430 3440

caagatgaatcgctggcccgcgaggtggataatgtcctgc 3440
agttggagtatgacaaagtggagatcctaaggacagtttt 3480
tctgcgtagccgaaagcgggccaaggacctaatcagtgag 3520
cagctgcgtgagtttcagcagaagcgcaccgccggcctgg 3560
gaaccatttacggacccacggacgacaagctggccgaggc 3600

3610 3620 3630 3640

gaagacggataagctaagggagcaaatcatcgacaaatat 3640
ttaatgcccaatcttcacgcgcttattgaggatgagaacg 3680
gttcaccgccggaggatgtacgcaaggtggcgttgtgttc 3720
tgctctttccaccgtcatctaccgtattttcaacactcgt 3760
ccgcctccaagcagcatcgttgagcgggtccatcactttg 3800

3810 3820 3830 3840

tgagcagggacaagagtttcaagtcgcgtattatgggcaa 3840
aaatcgcaagatgaatgttcgtggtcatccattggtattg 3880
cgtcaatactatgaagtgacgcactgcaatcattgtcaga 3920
cgattatctggggcgtgagcccgcaaggttatcattgtac 3960
agactgtaaattgaacatacaccgtcagtgctcgaaagta 4000

4010 4020 4030 4040

gtggacgagagttgtcccggtcccttgccccaggcaaaac 4040
gtctcgcccacaacgacaagatcagtaaattcatgggcaa 4080
aattcgaccgcgtaccagcgacgtcattggaaatgaaaag 4120
cgaagtcgtcaagatgaggaattggatgttgagttgactc 4160
cagaccgtggtcaggcgtcgattgtgcgtcaaccctctga 4200

FIGURE 17 cont.

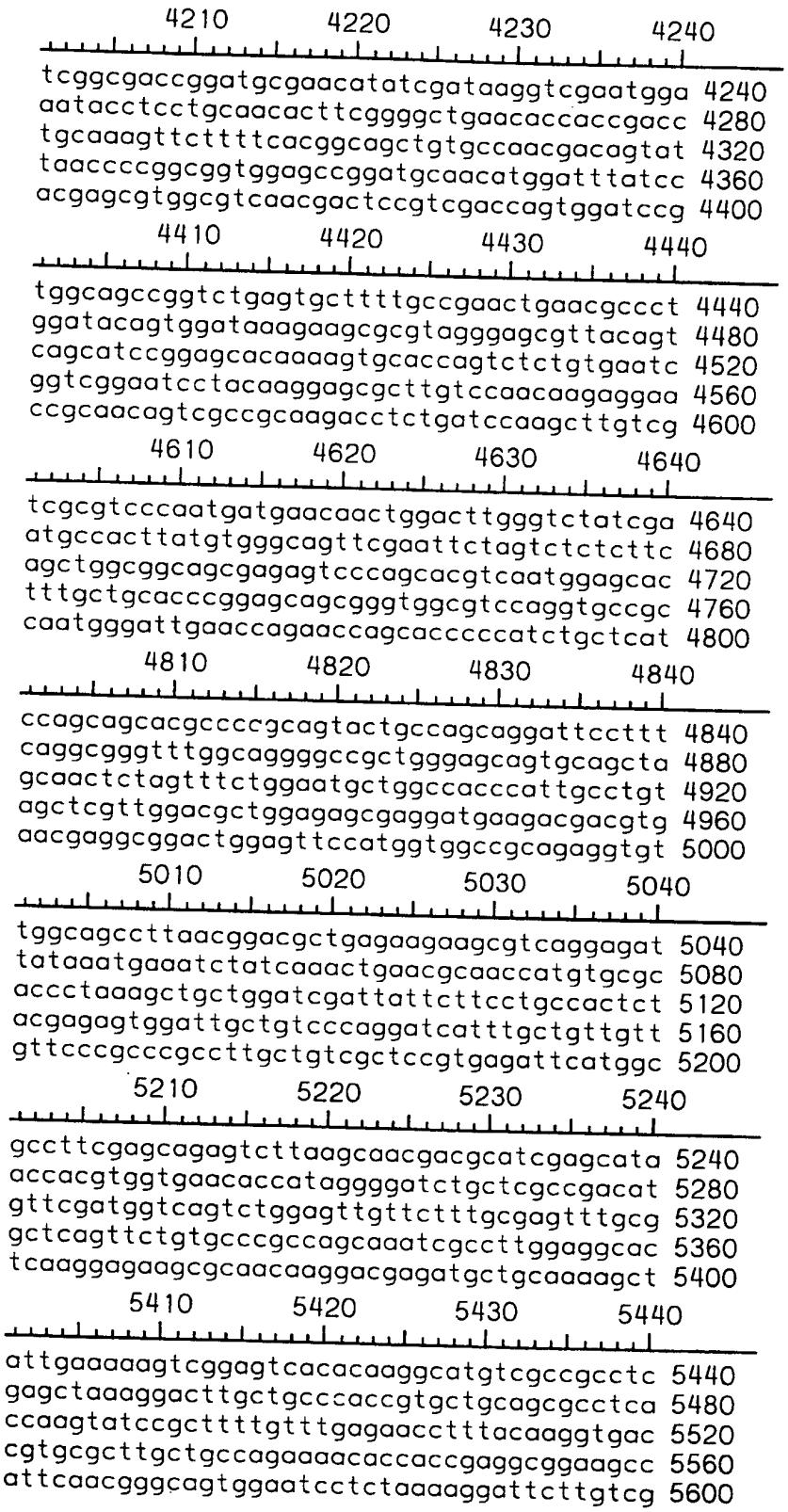

FIGURE 17 cont.

5610 5620 5630 5640

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gacaaggaagagtttaagaaattggacctgacccaacatc 5720
acctcatccatgacggcaatctgacgatcaagaagaatcc 5760
tagcgtgcagctacatggacttctgtttgaaaacatgatt 5800

5810 5820 5830 5840

gttttgctgaccaagcaggatgataaatattatctaaaga 5840
acttgcacaccccgctatcgatcaccaataagccagtcag 5880
tccaattatgagcattgatgcggacactttgatccggcag 5920
gaggcggctgataaaaattccttttcctcatcaagatga 5960
agacatcacaaatgttggagctacgcgcgcctagtagctc 6000

6010 6020 6030 6040

ggagtgcaagacatggtttaaacacttctcggatgtggct 6040
gctcgtcagtctaagaatcgttcaaagaacgcatcaagca 6080
accatgacacgagtattagtgatccagctctcgccgctat 6120
tccgcattccaacaccaaagagtcgttggagttgagcact 6160
gatacagtacagccattggctgcgacagccacattgacta 6200

6210 6220 6230 6240

ccacaccattggccccaatgctgcctatagccacggttac 6240
accggctccagcgaccaataatagtaacgttagctctctt 6280
actggagttcagttgcgaaaccctcaacgggatgcgacag 6320
caagtgaatctgatgcggattatgtaaacacaccaaagcc 6360
gcgttcgagccaaaatgaagttaatcgcactatgtccata 6400

6410 6420 6430 6440

agaagcactggcgaacccattcagaagtattcggcgaatg 6440
ggacggaagcaaacgacgttactttacgacactctcagtc 6480
gactagggaatcggttagaccaggatctactggggaggag 6520
cgaaactccacgtatggtatggttggaggtaactccaaac 6560
gcgacagcgccagcattgtctgctcgaacaactcgaacaa 6600

6610 6620 6630 6640

cacgcgcacccttctgatgcagagtcccttggtggaccct 6640
acggccattcaggtcagcattagtcccgctcacacagcgg 6680
aacctgtgttgacaccaggagagaagttacgccgcttgga 6720
cgcctccattaggaatgatttgctggagaagcagaaaatc 6760
atttgtgatatcttccgtttgccagtggaacactacgacc 6800

6810 6820 6830 6840

agattgtggacattgccatgatgccagaggcgccgaaaga 6840
cagtgcagatattgctttagctgcttacgatcagattcaa 6880
accctgaccaagatgctgaacgagtacatgcacgtcacgc 6920
ctgagcaagaggtctcagcggtgtccacggcggtttgtgg 6960
ccactgtcacgagaaggaaaagcttcgtaagaaggtggca 7000

FIGURE 17 cont.

```
          7010        7020        7030        7040
ccatcctcatcgttttcttcatcaccaccaccgctgccgc 7040
ctcccaataggcagcatgcccaggctcaggcccagatacc 7080
gccatcgcggctgatgcccaaactacaaactcttgatctt 7120
gacgaagttgccatacacgaagacgatgacggatactgtg 7160
agatcgacgaactgcgcttaccggctattccgtccaaacc 7200
          7210        7220        7230        7240
acatgagcggcccacaacgccactggctcctttcaatact 7240
gagccgaaaacttcacaatctgttatagatgcctcgaaac 7280
gtcaatccactgatgccgttccggagggattactggaaca 7320
agaaccactcgaaggcgataagacggagaccaagggtgaa 7360
gataatgaagtgaaaactgtgccgtcagataagctaagtg 7400
          7410        7420        7430        7440
aatcatgcaatgaagagaggcaatgtgtggaggcggatat 7440
cacaaaggaagtggcagatccaacgacctctaagaatgaa 7480
gctgcagcatcggtggatgaattaccaagccagagccggg 7520
agataaaaacggctgaaaacgcaagcaaatctgtagctga 7560
caaaaaggaagacaacgaggaaaccatcgaagaaggtgtg 7600
          7610        7620        7630        7640
gcatccacggtcgatagctccactcaaacatcaccaactg 7640
aatcgccgaaagagccggataagttaactggaggatcgag 7680
cagcacctgtgggccgaatcgcattcagcacgctagtgtg 7720
ctggagccgagtgtgccctgccatgcactcagcagcattg 7760
taacaatactgaatgagcagatttccatgcttttgccaaa 7800
          7810        7820        7830        7840
aattaacgaacgcgatatggaaagggagcgattgcgtaaa 7840
gagaatcaacaccttcgcgagctcttgagtgcgctgcatg 7880
atcgacagcgagttgatgaagtaaaggaaactccgtttga 7920
tctaaagaagctgatgcatgctgaggatgtagagtttgac 7960
gatgatattgacgccatttccaacagttcgctgacgccaa 8000
          8010        8020        8030        8040
cgcctacgccgattcccacagcatcaccaagcgccagcgg 8040
ccaggtagagacagcggaagccatgaggattactagcact 8080
gaggatgaggaataggaaacttgtttaatttttttttgta 8120
taaaatacatatttgttcttgttttcccgcacgcatttg 8160
accaattgaattggcggcgtctatgttttacgagtagcct 8200
          8210        8220        8230        8240
ttcttagagcatgccttttactactgttaatgtagcttca 8240
tttgttttcttaaaatattatgtattccacaagagccgat 8280
aacactttaaattaagtctaacattgtcccctcgtaaaac 8320
taattaaatacatatatatatgcatattatcgacttaaaa 8360
aatatcagataaacacacaatgtttgatacatttataaag 8400
```

FIGURE 17 cont.

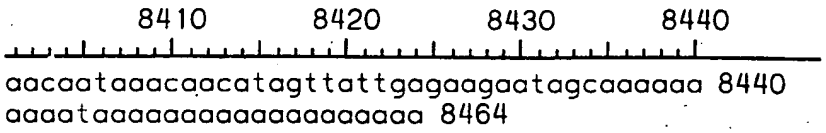

FIGURE 17 cont.

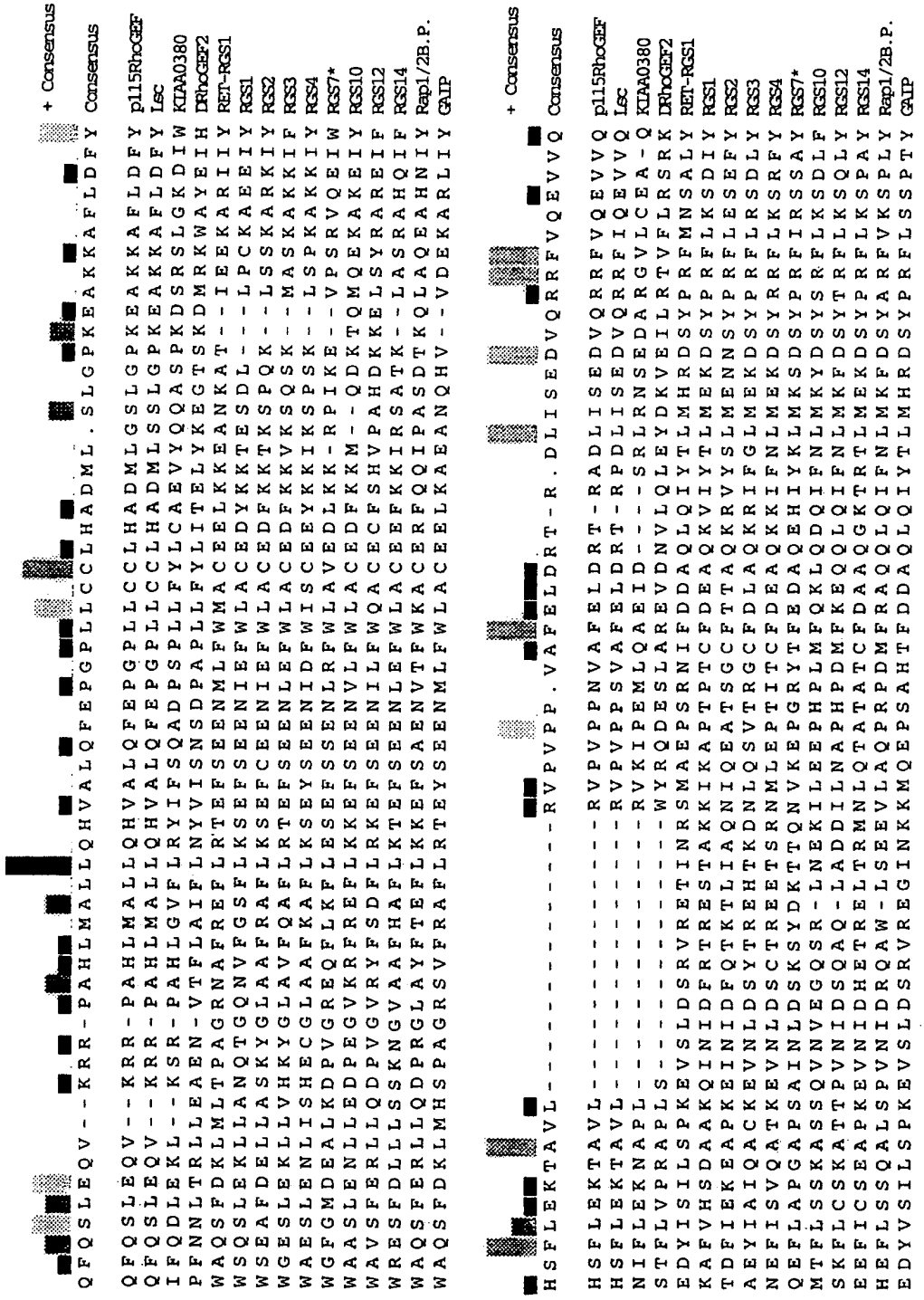

FIGURE 18

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(57) Abstract

Monomeric GTPase guanine nucleotide exchange factor (GEF) have been identified which also contain an RGS region analogous to those of GTPase activating proteins (GAP). One of these GEF proteins, a Rho GEF has been demonstrated to contain an RGS sequence that has GAP activity toward a α subunit of a heterotrimeric G prote in.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/06051

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C07K14/82 A61K38/17 G01N33/68 C12N15/63
C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MATTHEW J. HART ET AL.: "Identification of a novel Guanine nucleotide exchange factor for the Rho GTPase" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 41, 11 October 1996 (1996-10-11), pages 25452-25458, XP002057776 MD US cited in the application the whole document --- -/--	1-28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
21 September 1999	05/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DAVID M. BERMAN ET AL.: "GAIP and RGS4 are GTPase-activating proteins for the Gi subfamily of G protein alpha subunits" CELL, vol. 86, no. 3, 9 August 1996 (1996-08-09), pages 445-452, XP002111126 NA US cited in the application the whole document ---	1-28
P,X	KOZASA T ET AL: "p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13 'see comments!." SCIENCE, (1998 JUN 26) 280 (5372) 2109-11. , XP002111127 abstract page 2109, right-hand column, paragraph 2 -page 2111, middle column, paragraph 2 ---	1-28
P,X	ALAN HALL: "G proteins and small GTPases: Distant relatives keep in touch" SCIENCE, vol. 280, 26 June 1998 (1998-06-26), pages 2074-2075, XP002111128 DC page 2074, middle column, paragraph 2 -page 2075, right-hand column, paragraph 2 ---	1-28
P,X	HART M J ET AL: "Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13 'see comments!." SCIENCE, (1998 JUN 26) 280 (5372) 2112-4. , XP002111129 the whole document ---	1-28
P,X	MAO J ET AL: "Guanine nucleotide exchange factor GEF115 specifically mediates activation of Rho and serum response factor by the G protein alpha subunit Galpha13." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 OCT 27) 95 (22) 12973-6. , XP002111130 abstract page 12974, right-hand column, paragraph 2 -page 12976, right-hand column, paragraph 1 --- -/--	1-29

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/06051

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 98 20127 A (ONYX PHARMACEUTICALS) 14 May 1998 (1998-05-14) page 2, line 13 -page 9, line 32 page 19, line 13 -page 23, line 2 page 36, line 8 -page 37, line 32 ---	19-25
P,X	WO 98 57990 A (ONYX PHARMACEUTICALS) 23 December 1998 (1998-12-23) page 3, line 16 -page 4, line 2 page 4, line 19 -page 5, line 13 page 6, line 20 -page 7, line 3 page 8, line 26 -page 9, line 12 page 19, line 14 -page 20, line 11 page 20, line 24 -page 24, line 30 ---	19-25
T	FUKUHARA S ET AL: "A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho." JOURNAL OF BIOLOGICAL CHEMISTRY, (1999 FEB 26) 274 (9) 5868-79. , XP002111131 abstract page 5873, left-hand column, last paragraph -page 5874, right-hand column, paragraph 3 page 5875, right-hand column, paragraph 2 -page 5878, right-hand column, last paragraph -----	

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INTERNATIONAL SEARCH REPORT

Ir ational application No.
PCT/US 99/06051

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 18 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

.formation on patent family members

Interr··nal Application No
PCT/US 99/06051

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9820127 A	14-05-1998	AU 4801097 A	29-05-1998
WO 9857990 A	23-12-1998	AU 7966498 A	04-01-1999